

The Effect of Acute Docosahexaenoic Acid Intake on Left Ventricular Function and
Susceptibility to Ischemia-Reperfusion Injury in the Isolated Rat Heart

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The chronic intake and myocardial incorporation of n-3 highly unsaturated fatty acids (HUFA), particularly docosahexaenoic acid (22:6n-3, DHA), is believed to protect against ischemia-reperfusion injury, possibly through oxidative stress-dependent upregulation of enzymatic antioxidants. These adaptations may occur as early as 24 hours after the initial intake, but the effect of acute (<24 hour) DHA intake on myocardial DHA accretion, susceptibility to ischemia-reperfusion injury, and content of enzymatic antioxidants has not been characterized. Additionally, recent research suggests that acute intake patterns may be the norm in human populations, and thus it is important to study the effect of this dietary pattern on cardiovascular physiology. Therefore, Sprague-Dawley and Wistar rats were exposed to a one time gavage of a high DHA oil (DHASCO, 0.4 mg DHA/g body weight) to study the effect of acute DHA intake on left ventricular function and susceptibility to ischemia-reperfusion in the Langendorff *ex vivo* rat isolated heart model. Left ventricular myocardial samples were collected and analysed for total fatty acids, neutral lipid composition by thin layer chromatography, lipidomics by HPLC MS-MS, and the enzymatic antioxidants MnSOD, CuZnSOD, and GPx by western blotting. A single dose of DHA affects the fatty acid profiles of both plasma and left ventricle at 24 hours. The concentration of both eicosapentaenoic acid (EPA) and DHA is increased in plasma total lipids, with a corresponding decrease in the relative percent of the n-6 HUFA arachidonic acid (ARA) and adrenic acid ($p<0.05$). EPA is additionally elevated in the total lipids of the left ventricle ($p<0.05$), with a non-significant trend towards increased DHA ($p=0.053$). However, left ventricular phospholipid and triacylglycerol DHA concentrations are significantly elevated 24 hours following DHA treatment ($p<0.05$). Lipidomic analysis demonstrated a trend towards increasing total DHA ($p=0.081$) and DHA in phosphatidylethanolamine (PE, $p=0.078$) fractions,

primarily driven by increases in 16:0/DHA PE, although this did not reach statistical significance. This altered lipid profile is associated with reduced baseline left ventricular function in Sprague-Dawley male rats ($p < 0.05$), but improved post-ischemic left ventricular functional recovery ($p < 0.05$) and reduced infarction ($p < 0.05$ when males and females were combined) in a constant flow isolated heart model. An effect of DHA treatment on left ventricular function was not observed in Sprague-Dawley female rats ($p > 0.05$). DHA-fed male Wistar rats perfused with constant pressure had higher coronary flow during reperfusion ($p < 0.05$), but DHA treatment in these animals did not reduce infarction ($p > 0.05$), indicating that the effect of acute DHA treatment on ischemia-reperfusion injury is dependent on animal strain and the method of heart perfusion. There was no effect of acute DHA feeding on the myocardial protein content of the enzymatic antioxidants CuZnSOD, MnSOD, and GPx ($p > 0.05$). Therefore, a single dose of DHA increases plasma and myocardial EPA and DHA content at 24 hours and affects myocardial and vascular function at 24 hours dependent on strain and sex. This effect is independent of increases in the myocardial protein content of the enzymatic antioxidants tested.

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List of Abbreviations

4-HNE	4-hydroxy-2-nonenal
4-HXE	4-hydroxy-2-hexenal
ARA	arachidonic acid
ATP	adenosine triphosphate
AUC	area under the curve
BHT	butylated hydroxyl toluene
COX-2	cyclooxygenase 2
DAG	diacylglycerol
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EPA	eicosapentaenoic acid
FABP	fatty acid binding protein
FABP _{pm}	plasma membrane associated fatty acid binding protein
FAT/CD-36	fatty acid translocase
FATP	fatty acid transport proteins
GCLC	glutathione transferase A1
GSTA1	γ -glutamylcysteine ligase catalytic subunit
GSTA2	glutathione-S-transferase A2
HO-1	hemoxygenase-1
HSP	heat shock proteins
HUFA	highly unsaturated fatty acid
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acids
n-3	omega – 3
n-6	omega – 6
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf-2	nuclear factor-like 2
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PLB	phospholamban
PKC θ	protein kinase C theta

PMCA	plasma membrane Ca^{2+} ATPase
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
RBC	red blood cell
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
TAG	triacylglycerols
TBARS	thiobarbituric acid reactive species
TLE	total lipid extracts
TLR4	toll-like receptor 4
TNF- α	tumour necrosis factor alpha
TTC	2,3,5-triphenyltetrazolium chloride
xCT	Xc ⁻ high affinity cystine transporter

Chapter 1

Introduction

The n-3 highly unsaturated fatty acids (HUFA, ≥ 20 carbons, ≥ 3 carbon-carbon double bonds) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been studied extensively in relation to their benefit to cardiovascular health (Mozaffarian & Wu 2011). Increased dietary intake (Burr et al., 1989; GISSI, 1999) and elevated blood levels (Albert et al. 2002) of n-3 HUFA are associated with reduced cardiovascular disease mortality in humans, but recent meta-analyses of clinical interventions have indicated that prescribing an increased intake of n-3 HUFA may not reduce cardiovascular events (Kwak et al. 2012; Rizos et al. 2012), casting doubt on their clinical efficacy. Although cardioprotective effects of n-3 HUFA have been observed in myocardial cell culture (Xiao et al. 1997), *ex vivo* (Zeghichi-Hamri et al. 2010), and *in vivo* (McLennan et al. 1993) animal models, the understanding of the effect of n-3 HUFA on molecular and physiological pathways of the cardiovascular system, including the heart, is incomplete (Mozaffarian & Wu 2011). It is therefore essential to further the mechanistic understanding of the role n-3 HUFA in order to increase their clinical utility in the prevention and treatment of cardiovascular disease.

The role of n-3 HUFA in cardiovascular disease is complicated by the fact that they affect the heart through numerous mechanisms (Mozaffarian & Wu 2011). This includes modulation of myocardial ion channels (Xiao et al. 2001), changes in the profiles of bioactive lipid mediators (Kelley et al. 1999), and through their structural role in plasma and mitochondrial lipid membrane biophysics (Gawrisch et al. 2003). These mechanisms may, individually or in sum, contribute to observed reductions in arrhythmias (McLennan et al. 1985), infarction (Castillo et

al. 2013; Zeghichi-Hamri et al. 2010) and increases in post-ischemic blood flow (Force et al. 1989) that are associated with n-3 HUFA treatment in animal models. However, n-3 HUFA also increase the oxidative stress that the myocardium is subjected to (Castillo et al. 2013), both through their generation of reactive oxygen species (ROS) (Aires et al. 2007) and their susceptibility to lipid peroxidation (Saito & Kubo 2003). Although ROS contribute to ischemia-reperfusion injury (Kim et al. 2006; Temsah et al. 1999), they also serve as a stimulus to increase the hearts resistance to future oxidative stress through a phenomenon known as preconditioning (Bolli 2000). Late phase preconditioning protects the myocardium from ischemia-reperfusion injury from 24-72 hours after the preconditioning stimulus, and depends on the *de novo* synthesis of protective proteins, including enzymatic antioxidants (Bolli 2000). Recent studies (Abdukeyum et al. 2008; McGuinness et al. 2006), as well as investigations in our lab (Smith et al. 2012), have suggested that n-3 HUFA may act like preconditioning agents, possibly through oxidative stress pathways. In support of this, chronic dietary intervention with n-3 HUFA has been shown to increase cellular antioxidant enzymes (Jahangiri et al. 2006) and, importantly, to afford protection from ischemia-reperfusion injury (Castillo et al. 2013). However, currently no information exists regarding the potential of acute n-3 HUFA intake to effect adaptations in left ventricular function and ischemic susceptibility in the late phase window (24-72 hours) of preconditioning. This is particularly relevant given the acute intake patterns observed in humans (Patterson et al. 2014), and may help to explain the discordance between positive laboratory findings and their translation to cardioprotection in clinical populations. Important sex differences in n-3 HUFA metabolism (Kitson et al. 2012) and susceptibility to ischemia-reperfusion injury (Bae & Zhang 2005) also exist which may influence the cardiovascular effects of acute n-3 HUFA intake and should therefore be characterized.

This thesis therefore aims to investigate the effect of acute DHA intake on the left ventricular function and ischemic susceptibility of the isolated rat heart 24 hours after a single DHA feeding. It was necessary to complete preliminary method development, which included examining a time course for ischemia prior to reperfusion and sex differences in response to DHA exposure, and considerations in regard to rat strain and isolated heart perfusion protocols, as these factors affect the myocardial response to ischemia-reperfusion injury. The DHA treatment involved oral gavage and was compared with a soybean oil control. In addition to left ventricular function and heart infarction, levels of DHA in plasma and left ventricle, as well as the protein content of enzymatic antioxidants, were measured.

Chapter 2

Biochemical and Physiological Foundations

Understanding the effect of acute DHA intake on ischemia-reperfusion injury in the isolated heart requires insight into not only DHA absorption and metabolism, but also understanding of the processes of both cardiac function and ischemia-reperfusion injury and the models used in their study, with particular focus on the Langendorff. Recent evidence has suggested the potential of DHA to impact the heart through induction of oxidative stress, alteration of lipid mediator signalling, and modulation of cell membrane structure and function. Importantly, the form of DHA, the feeding protocols (i.e. acute vs. chronic dietary interventions), and the sex of animals tested have important implications for the effects on cardiovascular function and susceptibility to ischemia-reperfusion injury and, therefore, must also be considered.

2.1. The Langendorff Isolated Heart

A common model used to study cardiovascular physiology is the Langendorff isolated heart. Developed in 1895 by Oscar Langendorff, the isolated heart has facilitated the discovery of numerous aspects of cardiovascular physiology, and in modified forms it continues to be used to this day. This well-established model has the advantage that, in isolating the heart, it eliminates the potentially confounding influences of neural and hormonal factors, making it ideal for the mechanistic study of the myocardium (Skrzypiec-Spring et al. 2006).

In the Langendorff preparation, the heart is excised, cannulated via the ascending aorta, and perfused in retrograde. The perfusion solution enters the coronary arteries via the ostia, and, following the perfusion of the myocardium, exits via the coronary sinus and right atria (Bell et al.

2011). With this supply of oxygen and metabolic substrates, the heart is able to maintain contractile function for several hours. Several perfusion solutions have been employed in the Langendorff model, including donor blood from another animal, buffers supplemented with isolated red blood cells, and bicarbonate-buffer based physiological solutions, although the latter is the most common in the literature.

The supply of perfusion solution to the Langendorff isolated heart can be maintained with either constant pressure or constant flow, and the method used impacts heart function. In constant flow perfusion, a peristaltic pump is used to deliver a constant amount of perfusion solution based on the estimated coronary flow of the experimental animal (Bell et al. 2011). Using this method has the advantage of delivering a known, constant volume of perfusion solution to the heart. However, with constant flow the coronary vasculature is unable to regulate flow as it does *in vivo*. Conversely, in constant pressure perfusion the hydrostatic pressure of the perfusion solution is kept constant, allowing the coronary vasculature to control the flow rate. For this reason, constant pressure perfusion is considered to be more physiologically relevant (Skrzypiec-Spring et al. 2006).

2.2. Myocardial Ischemia-Reperfusion Injury

Ischemia is the absence of blood flow to a tissue which results in reduced oxygen supply, substrate delivery, and metabolite removal (Murphy & Steenbergen 2008). Reperfusion, or the restoration of blood flow to ischemic tissue, is required in order to salvage myocardial cells, but, counterintuitively, is also itself an injurious process (Hoffman et al. 2004). Ischemia-reperfusion injury adversely affects the force generation (Bolli & Marbán 1999) and relaxation (Piper et al. 2003) of the myocardium, due in part to impairment of myocardial Ca^{2+} sensitivity (Van Eyk et

al. 1998; Gao et al. 2014) and SERCA function (Temsah et al. 1999). Additionally, myofilament contracture, defined as sustained shortening of the myocardium, reduces myocardial relaxation (Piper et al. 2003) and increases cardiomyocyte susceptibility to mechanical damage (Schlüter et al. 1996). Significant portions of the myocardium may also be lost to infarction, which is caused by apoptotic and necrotic cell death (Kalogeris et al. 2012), as well as autophagy (Hamacher-Brady et al. 2006).

There are a number of metabolic changes that occur during myocardial ischemia-reperfusion which contribute to the observed functional impairment and infarction. Oxidative metabolism is arrested in the ischemic myocardium, resulting in the depletion of cellular high-energy phosphates, with levels of creatine phosphate falling more rapidly than ATP (Murphy & Steenbergen 2008). With increased anaerobic glycolysis, as well as ATP breakdown (Smith et al. 1993), cellular pH also drops rapidly in the ischemic heart (Murphy et al. 1991). Depletion of ATP affects the functioning of ATP-dependent enzymes and ion channels, resulting in increased levels of cytosolic Na^+ (Malloy et al. 1990; Pike et al. 1990) and Ca^{2+} (Steenbergen et al. 1987; Imahashi et al. 2005).

Furthermore, ischemia-reperfusion injury results in increases in myocardial oxidative stress, which has been implicated as a major contributor to the myocardial functional impairment and damage observed (Kim et al. 2006; Temsah et al. 1999). ROS are highly reactive molecules and atoms containing oxygen, which have the potential to abstract protons from lipids, proteins, and DNA resulting in cellular damage (Freeman & Crapo 1982). ROS are produced by the myocardium both during ischemia (Becker et al. 1999) and reperfusion (Kevin et al. 2003), although the amount of ROS produced during reperfusion is greater. The primary sources of superoxide (O_2^-), the predominant ROS produced early in ischemia-reperfusion, are electron

transport chain enzymes, including cytochrome p450 oxidases, and oxidation enzymes such as xanthine oxidase (Kalogeris et al. 2012; Zweier & Talukder 2006). If they are not neutralised by the cell's innate oxidative stress handling systems, ROS in the myocardium are pro-arrhythmic (Opie 1989), alter intracellular Ca^{2+} levels (Aires et al. 2007), induce inflammatory responses (Rodrigo et al. 2013), and cause the mitochondrial permeability transition pore to open (Halestrap et al. 2004), resulting in apoptotic and necrotic cell death (Gottlieb et al. 1994; Reeve et al. 2007).

2.3. Cardiac Excitation-Contraction Coupling

Cardiac excitation-contraction coupling is defined as the series of physiological processes occurring from electrical excitation of the cardiomyocyte to Ca^{2+} -induced contraction of the myofilaments (Bers 2002). L-type Ca^{2+} channels, located at the sarcolemmal-sarcoplasmic reticulum (SR) junction, open in response to an action potential resulting in Ca^{2+} influx (Meissner & Lu 1995). This in turn activates SR Ca^{2+} release through ryanodine receptors (RyR) resulting in an increase in cytosolic Ca^{2+} concentrations (Meissner & Lu 1995). Cytosolic Ca^{2+} binds to the myofilament regulatory protein troponin C, causing dissociation of tropomyosin from the actin filaments (Gordon et al. 2000). This reaction frees the myosin binding sites on the actin molecule allowing cross bridge cycling, and a myofilament contraction, to occur (Gordon et al. 2000). The force of the cardiac contraction (contractility) is dependent on both the cytosolic Ca^{2+} concentration and the sensitivity of the myofilaments to the Ca^{2+} signal (Bers 2002).

Relaxation of the myocardium requires that Ca^{2+} dissociate from troponin C, thus allowing tropomyosin to occupy its position on the actin molecule blocking myosin binding sites and cross

bridge cycling (Bers 2002). For relaxation to occur, Ca^{2+} concentrations in the cytosol must be reduced by the concerted action of several transport proteins including the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA), $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), plasmalemmal Ca^{2+} ATPase (PMCA), and the mitochondrial Ca^{2+} uniporter (Bers 2002). The latter 2 proteins are referred to collectively as the slow systems, and contribute minimally to Ca^{2+} removal (Bassani et al. 1994). In rats, SERCA handles the majority (~92%) of Ca^{2+} removal, with the balance of Ca^{2+} removal proceeding through forward action of NCX (~7%) and slow systems (~1%) (Bassani et al. 1994).

The Langendorff isolated heart can be instrumented in order to measure the effect of ischemia-reperfusion injury on cardiac excitation-contraction coupling. Systolic pressure is the peak of the left ventricular pressure waveform and describes the highest amount of force that the myocardium develops within a given beat. Diastolic is used to measure contracture, an indicator of ischemia-reperfusion injury caused by myocardial ionic disturbances. Contractility, or developed pressure, is the difference between systolic and diastolic pressure and is an indicator of the heart's ability to generate force. Contractility is dependent on both the Ca^{2+} levels in the cytosol, and the sensitivity of the myofilaments to Ca^{2+} . The rates of contraction and relaxation are derivatives of the left ventricular pressure waveform. Rate of contraction is dependent on excitation-contraction coupling and myofilament Ca^{2+} sensitivity. The rate of relaxation is primarily determined by the activity of SERCA in sequestering Ca^{2+} in the SR. The responses of these variables to the nutritional intervention and ischemia-reperfusion protocol provide insight into the specific physiological changes occurring in the myocardium.

2.4. Effects of N-3 HUFA on Cardioprotection

The n-3 HUFA EPA and DHA affect numerous biological pathways and systems, which complicates the understanding of their mechanistic role in cardiovascular disease. Although EPA and DHA are traditionally found together in dietary sources, and have often been studied in combination, they may have differential effects on the cardiovascular system (Cottin et al. 2011). Following dietary intake, EPA and DHA are differentially incorporated into the plasma (Hodge et al. 1993) and tissues (Frøyland et al. 1996). Additionally, supplementation with DHA, but not EPA, affects hemodynamics and heart rate in humans (Mori et al. 1999). As compared with EPA, DHA is more susceptible to peroxidation (Saito & Kubo 2003), indicating that it may increase oxidative stress to a greater extent than EPA. Therefore, EPA and DHA should be studied independently in order to gain a complete understanding of their mechanistic effects.

Chronic dietary supplementation with EPA + DHA has been found to increase myocardial resistance to ischemia-reperfusion injury (Castillo et al. 2013; Zeghichi-Hamri et al. 2010; Abdukeyum et al. 2008) and to improve post-ischemic blood flow (Force et al. 1989). These studies employ high dose gavage or modified chow diets to effect increases in myocardial phospholipid n-3 HUFA, while simultaneously displacing the predominant n-6 HUFA arachidonic acid (ARA, C 20:4n-6). Although the altered myocardial fatty acid profile appears to be cardioprotective in ischemia-reperfusion injury, the specific mechanisms involved have not been fully elucidated. Increased n-3 HUFA in myocardial phospholipids following chronic dietary intervention may affect several parameters and pathways relevant to ischemia-reperfusion injury including membrane structure and function, fatty acid-derived eicosanoids, and oxidative stress pathways (discussed in detail in sections 2.5, 2.6, and 2.7 respectively).

There is also evidence for cardioprotective effects of free n-3 HUFA administered acutely during an ischemia-reperfusion protocol, indicating that n-3 HUFA can act independently of their incorporation into myocardial phospholipids. Acute infusions of DHA immediately following an ischemic stress result in reductions in infarction (Smith et al. 2012); acute n-3 HUFA treatment has also been shown to reduce arrhythmias in isolated rat cardiomyocytes (Kang & Leaf 1994) and *in vivo* animal models (McLennan et al. 1993). As n-3 HUFA are not incorporated into the myocardial membrane phospholipids in these experiments, these results are independent of phospholipid composition, and thus reflect an acute effect of free n-3 HUFA. These near immediate effects on the heart primarily involve ion channel interactions, which are hypothesized to result in the observed anti-arrhythmic effects (Billman et al. 1997). N-3 HUFA interact directly with myocardial ion channels including the voltage gated L-type Ca^{2+} channel (Xiao et al. 1997) and the alpha subunit of the sodium channel (Xiao et al. 2001), which may contribute to observed reductions in myocardial excitability with acute n-3 HUFA treatment (Kang et al. 1995). It is likely a combination of the acute and chronic effects of n-3 HUFA that contributes to observed reductions in cardiovascular disease, although determining their individual mechanistic contributions, both in laboratory and clinical settings, has proven difficult.

2.5. Effects on Membrane Structure and Function

N-3 HUFA are found primarily in the sn-2 position of plasma membrane phospholipids. However, *de novo* phospholipid synthesis through the Kennedy pathway (Kennedy & Weiss 1956) results in phospholipids rich in monounsaturated and diunsaturated fatty acids in the sn-2 position, with little n-3 HUFA present. Instead, n-3 HUFA are incorporated into the plasma

membrane phospholipids through a remodelling process known as the Lands cycle (Lands 1958). Through a series of deacylation and reacylation reactions, the fatty acids esterified to the sn-2 position of individual phospholipids are altered from their *de novo* composition.

Due to the unique chemical structure and 3-dimensional configuration of DHA, its incorporation into phospholipids of plasma or organelle membranes alters their structure and function. Incorporation of DHA alters the biophysical properties of the membrane resulting in increases in membrane fluidity, ion permeability, lipid raft dynamics, and fusion (reviewed in Stillwell & Wassall, 2003). These biophysical changes, along with direct interaction of DHA with ion channels and transmembrane proteins, affect numerous biological processes carried out at the membrane (Lee et al. 2003; Wong et al. 2009).

Incorporation of EPA and DHA also affects the ion permeability and electrochemical gradient of the plasma membrane through modulation of ion channel kinetics. The n-3 HUFA affect lipid raft dynamics as previously discussed, and the ion channels that localize in these membrane microdomains are modulated by the specific lipid raft structure (reviewed in Dart, 2010). It is important to note, however, that most ion channel interactions are caused by free n-3 HUFA (discussed in section 2.4). During ischemia, the activity of plasmalogen-specific phospholipase A₂ is increased (Ford et al. 1991), resulting in cleavage of fatty acyls including n-3 HUFA from the sn-2 position of plasma membrane phospholipids. This pool of liberated n-3 HUFA could potentially exert anti-arrhythmic effects through the known interactions with ion channels demonstrated in acute cultured cardiomyocyte studies.

2.6. Lipid Mediators of Inflammation

The pathogenesis of cardiovascular disease is characterized by increased inflammation (Kain et al. 2014), and one of the pathways through which DHA is thought to ameliorate cardiovascular disease is through its role as a precursor to lipid mediator signalling molecules which have anti-inflammatory properties. The n-3 derived docosanoids have recently been identified as important mediators of inflammation resolution, controlling both the duration and magnitude of the inflammatory response (reviewed in Serhan, 2007). This class of pro-resolution compounds, termed specialized pro-resolving mediators, includes both resolvins and protectins. DHA is a precursor to protectins and D series resolvins. These compounds act to resolve inflammation through reduction of polymorphonuclear leukocyte migration and infiltration, cytokine production, and ROS generation (Serhan et al., 2000).

Lipid mediators can also be derived from the dominant n-6 HUFA ARA. When cleaved from membrane phospholipids by phospholipase A₂, ARA serves as a substrate for eicosanoid synthesis via enzymatic oxidation by cyclooxygenase, lipoxygenase, and cytochrome P450. Some of the eicosanoids derived from the predominant n-6 HUFA ARA, including leukotriene-B₄ and thromboxane-A₂, are pro-inflammatory and are thus considered to be detrimental to cardiovascular health (Mozaffarian & Wu 2011). DHA can compete in eicosanoid synthesis pathways by both displacing ARA in the membrane and directly inhibiting n-6 series eicosanoid synthesis, resulting in an overall decrease in production of these pro-inflammatory eicosanoids (Kelley et al. 1999).

2.7. N-3 HUFA and Oxidative stress

Lipid peroxidation is the redox process through which protons are abstracted from lipids by ROS, resulting in lipid radical products. These products are dangerously reactive and can damage cellular components and increase intracellular oxidative stress (Halliwell & Chirico 1993). The susceptibility of n-3 PUFA to lipid peroxidation is correlated with the number of double bonds in membrane phospholipids (Saito & Kubo 2003); therefore, a highly unsaturated fatty acid such as DHA may increase ROS-induced lipid peroxidation.

Chronic dietary intervention with n-3 HUFA has been correlated with increases in oxidative stress in several systems studied including isolated endothelial cells (Vossen et al. 1995), rat plasma (Song et al. 2000), liver cells (Farina et al. 2003), mouse kidney (Ibrahim et al. 1999), and human blood (Garcia-Alonso et al. 2012). The treatment with n-3 HUFA need not be chronic in order to elicit increases in tissue oxidative stress, as supply of high concentrations of n-3 HUFA has been shown to induce rapid ROS generation (Feng et al. 2012).

Although this oxidative challenge can induce apoptosis through ROS-mediated activation of the intrinsic apoptotic pathway (Diep et al. 2000; Aires et al. 2007), it may also serve to signal the myocardium to increase its oxidative stress handling capacity through increasing enzymatic antioxidants. One pathway involved may be the signalling of the n-3 derived lipid peroxidation product, 4-hydroxyhexenal (4-HXE) which upregulates enzymatic antioxidants (Nakagawa et al. 2014) through the transcription factor Nrf2 (Ishikado et al. 2013). Chronic n-3 HUFA feeding increases both 4-hydroxyhexenal levels (Anderson et al. 2012) and antioxidant enzymes in the murine heart (Anderson et al. 2012; Jahangiri et al. 2006; Castillo et al. 2013), and results in improved recovery following ischemia-reperfusion injury in isolated hearts (Castillo et al. 2013). Therefore, myocardial membrane incorporation of n-3 HUFA may induce ROS and reactive

aldehyde production below the level required for activation of the intrinsic apoptotic pathway, which may instead serve as a stimulus for upregulation of enzymatic antioxidants. However, no studies have tested the ability of n-3 HUFA to effect similar adaptations in the left ventricular function and ischemic susceptibility in an acute (<24 hour) time frame.

2.8. Role of ROS and Oxidative Stress in IR

Myocardial functional impairment and damage following ischemia-reperfusion injury is caused in large part by ROS (Kim et al. 2006; Temsah et al. 1999), and thus these molecules are a target for potential cardioprotective interventions. Increasing the heart's antioxidant defences through the addition of exogenous antioxidant defence enzymes has been shown to reduce infarction in some (Adlam et al. 2005; Bognar et al. 2006; Jones et al. 2003), but not all studies (Gallagher et al. 1986; Ooiwa et al. 1991; Vanhaecke et al. 1991), possibly due to differences in the specificity and cellular localisation of antioxidants used (Sheu et al. 2006). Therefore, the administration of exogenous antioxidants may not be a viable method for cardioprotection. However, increasing the endogenous antioxidant defences of the myocardium through a method known as preconditioning may be an alternative.

Preconditioning is defined as a stressor that elicits an adaptive response which attenuates damage from future stressors. An example occurs in the myocardium when it is exposed to brief periods of ischemia, as the heart adapts to become more resistant to future ischemic insults. Since the initial investigations by Murry *et al.* (Murry et al. 1986), this phenomenon, known as ischemic preconditioning, has shown promise in its ability to reduce many aspects of ischemia-reperfusion injury, including a reduction in apoptotic and necrotic cell death, reperfusion arrhythmias, and myocardial stunning (Pagliaro et al. 2001). Traditional ischemic

preconditioning employs short (<5 minute) bouts of ischemia before the main ischemic insult to induce cardio-protection (Penna et al. 2009), although other preconditioning stimuli elicit a similar cardioprotective effect, including exercise-induced (Chicco et al. 2007), heat stress (Yamashita et al. 1998), and pharmacological (Forbes et al. 2001) preconditioning. This myocardial preconditioning effect is ubiquitous in the animal kingdom, and has been demonstrated in all species studied (Nakano et al. 2000), including humans (Ikonomidis et al. 1997).

The protective effects of preconditioning exist in 2 distinct phases. The first, defined as early phase preconditioning, provides the heart with protection from ischemic insults within 2 – 3 hours of the preconditioning stimulus. The second, defined as late phase preconditioning, provides the heart with protection between 24 and 72 hours following the preconditioning stimulus (Penna et al. 2009). The mechanisms involved are separate and discrete in these 2 time periods, but the stimuli can be the same. Importantly, ROS activate pathways of both phases of protection (Sindram et al. 2002; Vanden Hoek et al. 1998), illustrating their central role in preconditioning the myocardial response to ischemia-reperfusion injury. When ROS scavengers are used during the ischemic preconditioning protocol the cardioprotective effect disappears (Sun et al. 1996; Baines et al. 1997). Interestingly, without an ischemic stimulus, hearts can be preconditioned solely with an increase in intracellular ROS; both pharmacologically generated ROS (Baines et al. 1997) and the addition of exogenous ROS (Tritto et al. 1997) induce a comparable preconditioning effect.

In response to a ROS preconditioning stimulus, the oxidative stress handling ability of the cell increases, which may allow the myocardium to survive future ischemia-reperfusion-induced ROS generation (Das et al. 1992; Rudiger et al. 2003). This adaptation occurs in the late phase

of preconditioning and is due mainly to increased *de novo* protein synthesis of protective antioxidant enzymes (Bolli 2000). The amount of enzymatic antioxidants increases 24 hours after ischemic preconditioning in dog hearts *in vivo* (Hoshida et al. 1993) and cultured rat cardiomyocytes (Yamashita et al. 1994), and this increase has been attributed to the ROS stimulus (Maulik et al. 1995). This increase in enzymatic antioxidants has been observed following pharmacological (Maulik et al. 1995), exercise induced (Yamashita et al. 1999), and heat stress-induced (Yamashita et al. 1998) preconditioning, and these changes correlated with reductions in susceptibility to ischemia-reperfusion injury. However, it is important to note that conflicting results have been reported *in vivo* using pig (Tang et al. 1997) and rabbit (Tang et al. 2000) models of ischemic preconditioning, suggesting that this mechanism may behave differently *in vivo*.

2.9. Effect of n-3 HUFA Intake on Blood and Tissue Levels

The blood and tissue levels of EPA and DHA are determined by the amount and frequency of their intake, and different tissues and blood pools respond to EPA and DHA intake in different ways. In the blood, the plasma fraction responds most rapidly to DHA intake (Metherel et al. 2009). EPA and DHA accretion also varies across different tissues (Rodrigues et al. 2014), and depends on both the accretion of EPA and DHA and their half-life within the tissue itself.

In animal studies, the intake of EPA and DHA results in blood and heart accretion, and this has been shown to occur as early as 72 hours following the initiation of intake in a study employing fish oil infusion (McGuinness et al. 2006). Other studies employing longer term dietary interventions with modified chow diets or daily gavage have similarly demonstrated

increased blood and myocardial EPA and DHA concentrations (Zeghichi-Hamri et al. 2010; Force et al. 1989; Castillo et al. 2013).

Intake patterns in humans differ, as the sources of EPA and DHA in an unsupplemented diet are typically consumed intermittently, although the regularity of EPA and DHA intake can be increased in individuals consuming supplements. However, adherence has been reported to be poor in study participants advised to increase EPA and DHA intake through regular supplementation (Patterson et al. 2014). It has been hypothesized that participants engage in compensatory supplementation patterns characterized by periods of decreased intake between visits and larger intakes immediately preceding assessment visits (Patterson et al. 2014). This pattern results in increases in plasma levels of EPA and DHA and erythrocyte levels of EPA. However, erythrocyte DHA remains depressed, as the inner membrane DHA pool takes longer to remodel to EPA and DHA intake whereas EPA remodelling of the outer membrane is rapid (Metherel et al. 2009). Such intake patterns may partly explain the negative results observed in clinical trials testing cardioprotection while employing fish oil supplementation (Kwak et al. 2012; Rizos et al. 2012).

In an acute feeding protocol it is therefore important to determine at what time, and in what molecular form, DHA enters the plasma and myocardium and can thus influence heart function. Postprandial plasma levels of n-3 HUFA in humans peak between 4 (Hanwell et al. 2009) and 6 (Harris et al. 2013) hours after ingestion, with levels declining, but remaining elevated relative to baseline, for at least 24 hours (Yang et al. 2012). The effect on the plasma phospholipid pool in humans is comparatively delayed, with DHA levels not reaching a peak until 24 hours after ingestion in this pool (Raatz et al. 2009).

The amount of DHA provided is an important consideration in order to ensure appreciable increases in plasma and left ventricle concentrations. In Sprague-Dawley rats the intake of 0.96 g of DHA per day for 8 weeks increased erythrocyte DHA, which has been considered as a marker for myocardial DHA accretion (Zeghichi-Hamri et al. 2010). This intake ranged from 0.64 to 0.27 mg/g body weight per day as the body weight of the animals increased, but the absolute DHA dose remained constant throughout the protocol.

2.10. Sex Differences in Cardiovascular Disease and n-3 HUFA

Sex differences in both ischemia-reperfusion injury (Bae & Zhang 2005) and DHA metabolism (Kitson et al. 2012) have been observed. Given initiatives and policies aimed at improving understandings of the effect of sex in health and disease (Clayton & Collins 2014; Health Canada 2009) it is important to examine the potential role of sex on the outcomes of this thesis.

Laboratory investigations in animals have demonstrated that females experience less ischemia-reperfusion injury in models of regional (Brown et al. 2005; Chicco et al. 2007; Edwards et al. 2009; Johnson et al. 2006; Song et al. 2003) and global (Bae & Zhang 2005) myocardial ischemia. Decreased oxidative stress (Barp et al. 2002) and reduced production of ROS (Lagranha et al. 2010) in female hearts have been observed, possibly contributing to this effect.

The effect of preconditioning is also sex-dependent, as pre-menopausal females experience less protection from infarction following both traditional ischemic (Song et al. 2003; Turcato et al. 2006) and exercise-induced (Chicco et al. 2007) preconditioning protocols. This effect

appears to be dependent on age, as 18 week old mice experienced preconditioning while 10 week old mice did not (Turcato et al. 2006).

Sex differences can also influence the content of n-3 HUFA in rat hearts. Chow-fed female rats have higher levels of DHA in several tissues including the heart as compared with males (Kitson et al. 2012). Sex hormones alter metabolism of fatty acids and DHA specifically as 17 β -estradiol (Kitson et al. 2012) and progesterone (Childs et al. 2012; Sibbons et al. 2014) have been implicated in increases in hepatic desaturases. This effect is observable in humans, as tracer studies have demonstrated increased conversion of α -linolenic to the longer chain products EPA and DHA acid in females as compared with males (Pawlosky et al. 2003; Burdge et al. 2002; Burdge & Wootton 2002).

Chapter 3

Rationale and Objectives

3.1. Rationale

N-3 HUFA are associated with reduced cardiovascular disease, and one of the cardioprotective mechanisms likely involves the reduction of ischemia-reperfusion injury. The role of n-3 HUFA in acute and chronic cardioprotection has been extensively studied, but recent studies (Abdukeyum et al. 2008; McGuinness et al. 2006) as well as investigations in our lab (Smith et al. 2012) have suggested that n-3 HUFA may also act like preconditioning agents, possibly through oxidative stress pathways. Late phase preconditioning protects the myocardium from ischemia-reperfusion injury as early as 24 hours after the preconditioning stimulus and depends on the *de novo* synthesis of protective proteins including enzymatic antioxidants. Currently no information exists regarding the potential of acute n-3 HUFA intake to effect adaptations in left ventricular function and ischemic susceptibility in the late phase window of traditional preconditioning. This is particularly relevant given the acute intake patterns observed in humans, and may help to explain the discordance between positive laboratory findings and their translation to cardioprotection in clinical populations. Important sex differences in n-3 HUFA content and oxidative stress capacity also exist which may influence the cardiovascular effects of acute n-3 HUFA intake and should therefore be characterized.

3.2. Statement of Objectives

This thesis aimed to understand the effects of acute DHA intake on myocardial function and susceptibility to ischemia-reperfusion injury. Parameters pertaining to the experimental model used, including type of perfusion, rat strain, and ischemic duration were first optimized prior to the investigation of acute DHA intake. The left ventricular contractile and coronary vascular functional response to acute DHA intake was then characterized in the Langendorff isolated heart model. Additionally, the potential role of DHA mediated cardioprotection through late phase preconditioning was investigated using an established ischemia-reperfusion protocol. Lipid analysis was employed in order to understand the dynamics of DHA intake and myocardial incorporation in this acute time frame. Comprehensive measures of the beat-by-beat left ventricular contractile parameters and the function of the coronary vasculature were made. Protection from ischemia-reperfusion injury was assayed using both the recovery of heart functional parameters and biochemical staining techniques. Measures of the protein content of enzymatic antioxidants in the left ventricle were made to determine the extent of antioxidant system adaptations.

3.3. Hypotheses

1. Increasing ischemia durations will result in an increasing degree of myocardial functional impairment and infarction
2. Acute intake of DHA will increase plasma and left ventricular myocardium DHA content
3. Rats supplemented with an acute dose 0.4 mg/g body weight of DHA 24 hours prior to the ischemia-reperfusion protocol will be less susceptible to myocardial functional impairment and infarction following ischemia-reperfusion
4. Females will respond less than males to acute DHA-mediated protection from ischemia-reperfusion injury
5. The protein content of the enzymatic antioxidants CuZnSOD, MnSOD, and GPx will be increased in myocardial samples from DHA treated animals relative to control

Chapter 4

Analytical Techniques

4.1. Animals, Diets, and Interventions

Animal procedures conformed to the standards set by the Canadian Council on Animal Care and were approved by the University of Waterloo Animal Care Committee. Sprague-Dawley and Wistar inbred rats were ordered from Harlan Laboratories (Mississauga, Ontario). Animals were group housed in the Department of Kinesiology facility in temperature and humidity controlled conditions on a reversed 12:12 hour light-dark cycle. Experimental animals were maintained on standard rat chow diets (Harlan Teklad 8640, fatty acid composition listed in Table 1) and had access to food and water ad libitum. In DHA feeding studies, animals were given a single oral gavage of 0.4 mg DHA/g body weight in the form of DHASCO oil (DSM, The Netherlands, fatty acid composition listed in Table 2). An equivalent volume of commercially available soybean oil was used as a control as soybean oil is the primary source of fatty acids in the Harlan Teklad 8640 diet, and therefore the control oil closely mirrored the fatty acid composition of the diet. Animals that were fed with an oral gavage 24 hours prior to isolated heart experiments were fed at a consistent time of day and subsequently transferred to individual cages to allow for fasting prior to experimentation.

4.2. Langendorff Isolated Heart Perfusion Protocol

Animals were anaesthetized by 1:3 sodium pentobarbital: 0.9% sterile saline administered by intra-peritoneal injection. Hearts were excised rapidly by median thoracotomy and placed into cold (<4°C) Krebs-Henseleit buffer briefly. Excised hearts were then cannulated in retrograde by the ascending aorta and fixed in place with silk sutures. A small incision was made in the left

atrium through which an intraventricular balloon catheter was passed to the floor of the left ventricle. The intraventricular balloon was then filled with degassed water and left ventricular diastolic pressure was set at 10 mmHg.

The heart was perfused with a modified Krebs-Henseleit bicarbonate buffer containing (in mM/L) 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 glucose, 2.5 CaCl₂, and 2.5 NaHCO₃. This was buffered to a pH of 7.40 at 37°C and oxygenated with 95% O₂ - 5% CO₂. Two distinct perfusion methods were used for the investigations in this thesis: constant flow (Chapter 5 & 6) and constant pressure perfusion (Chapter 7). In constant flow perfusion, a peristaltic pump and ultrasonic flow sensor are used to establish a constant rate of flow based on the animal's body weight and estimated coronary flow *in vivo*. The flow remains constant throughout the experiment, thus overriding the coronary vasculature's autoregulatory function. Conversely, in constant pressure perfusion, the hydrostatic pressure of the buffer reaching the heart is kept constant and the flow rate is controlled physiologically by the coronary vascular tone.

A 30 minute baseline period was used to allow cardiac function to normalize to the perfusion conditions. Exclusion criteria were established *a priori* as this is recommended for Langendorff isolated heart perfusion (Bell et al. 2011). They included a heart rate <175 beats per minute, developed pressure <50 mmHg, rate of contraction <2000 mmHg/s, and rate of relaxation >-1000 mmHg/s at baseline. Global ischemia was initiated by shutting off flow at the peristaltic pump and closing a valve proximal to the heart. Reperfusion was initiated following ischemia by opening the valve and re-establishing the flow rate using the peristaltic pump. Following the Langendorff protocol, hearts were removed from the system and placed immediately into cold (<4°C) Krebs-Henseleit buffer at a pH of 7.40. The aorta and atria were removed and hearts

were subsequently wrapped tightly in cellophane and aluminum foil and frozen in a -80°C freezer for further analysis.

4.3. Quantifying Myocardial Ischemia-Reperfusion Injury

Myocardial response to nutritional intervention and ischemia-reperfusion was determined by measuring the recovery of functional performance of the coronary vasculature and left ventricle, as well as biochemical assays of the myocardium. Parameters of heart function were measured continuously throughout the protocol (Powerlab/4SP and Chart version 5.5.1, ADInstruments, New Zealand), with baseline measures taken in the final minute of the 30 minute normalization period. Frozen whole heart samples were sliced transversely into 1 mm thick slices. These were incubated in a 1% w/v solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (88 mM Na₂HPO₄ , 1.8 mM NaH₂PO₄, pH=7.4) at 37°C for 20 minutes. TTC staining identifies viable tissue as active myocardial dehydrogenase enzymes produce red formazan-insoluble coloured pigments in a redox reaction (Schwarz et al. 2000). Unstained areas are assumed to lack dehydrogenase enzyme activity and are considered to be nonviable, infarcted tissue. Stained slices were visualized under a dissection microscope and images were captured using a digital camera. These were processed using ImageJ software (NIH public domain, <http://rsbweb.nih.gov/ij>) to quantify the unstained infarcted area as a percentage of the total myocardial volume.

4.4. Analysis of Fatty Acids

Fatty acids were analyzed as previously described (Metherel et al. 2012). Briefly, lipids were extracted from left ventricular myocardium samples and rodent chow diets according to the

Folch method (Folch et al. 1957). Samples were mixed with 2:1 chloroform methanol (v/v) containing 50 µg/ml butylated hydroxyl toluene (BHT) and docosatrienoic acid (C 22:3n-3) ethyl ester (Nu-chek Prep Inc., Elysian, MN, USA) as an internal standard for absolute concentration determinations and 500 µL of 0.2 M Na₂PO₄ buffer was added. Samples were inverted and centrifuged at 1734 *rcf* for 5 minutes and the organic phase was collected and total lipid extracts were stored at -80°C until further analysis.

Fatty acid methyl esters were prepared through the use of 14% BF₃ in methanol and heating at 85°C for 1 hour of isolated total lipid extracts (Morrison & Smith 1964). Direct transesterification procedures without prior lipid extraction were used to prepare fatty acid methyl esters from plasma and gavage oil. Fatty acid methyl esters were analyzed by gas chromatography using a Varian 3900 gas chromatograph (Varian Inc, Mississauga, ON, Canada) with a DB-FFAP capillary column with 15 m × 0.10 mm injected dose × 0.10 µm film thickness, polyethylene glycol capillary column (J & W Scientific, Agilent Technologies, Palo Alto, CA) with H₂ carrier gas flowing at 30 mL/min. Fatty acid concentrations were quantified through comparison with the internal reference standard docosatrienoic acid.

4.5. Statistical Analysis

Statistical analyses were performed using Graphpad Prism for Windows version 6.0 and SPSS for Windows version 11.5. Isolated heart vascular and left ventricular functional variables were compared using independent T-tests at individual timepoints. Area under the curve data was compared using 1 way ANOVA. *A priori* comparisons between functional variables across each time point were additionally analyzed with an unpaired t-test. Fatty acid data was analysed

by a multivariate general linear model. Statistical significance was determined at an α -level of 0.05.

Table 1. Fatty Acid Composition of Diet

Fatty Acid	µg Fatty Acid/mg diet	Percent of Total Fatty Acids in Diet
C 10:0	0.79 ± 1.75	nd
C 12:0	0.08 ± 0.03	0.13 ± 0.03
C 14:0	0.22 ± 0.04	0.33 ± 0.01
C 16:0	8.91 ± 1.53	13.46 ± 0.35
C 17:0	0.09 ± 0.01	0.14 ± 0.01
C 18:0	1.56 ± 0.26	2.47 ± 0.91
C 20:0	0.22 ± 0.02	0.34 ± 0.03
C 22:0	0.21 ± 0.01	0.33 ± 0.06
C 23:0	0.04 ± 0.01	0.06 ± 0.01
C 24:0	0.13 ± 0.01	0.20 ± 0.06
SFAs	11.53 ± 1.40	17.54 ± 0.71
C 12:1	nd	nd
C 14:1	0.01 ± 0.01	0.01 ± 0.01
C 16:1	0.47 ± 0.25	0.72 ± 0.35
C 18:1n-7	0.94 ± 0.12	1.43 ± 0.06
C 18:1n-9	13.93 ± 2.13	21.10 ± 0.13
C 20:1n-9	0.20 ± 0.02	0.31 ± 0.03
C 22:1n-9	0.04 ± 0.01	0.06 ± 0.01
C 24:1n-9	0.02 ± 0.01	0.03 ± 0.01
MUFAs	15.61 ± 2.33	23.66 ± 0.43
C 18:2n-6	32.87 ± 4.93	49.80 ± 0.20
C 18:3n-6	0.02 ± 0.01	0.02 ± 0.01
C 20:2n-6	0.03 ± 0.01	0.05 ± 0.01
C 20:3n-6	0.02 ± 0.01	0.03 ± 0.01
C 20:4n-6	0.09 ± 0.01	0.13 ± 0.03
C 22:2n-6	0.02 ± 0.01	0.03 ± 0.01
C 22:4n-6	0.02 ± 0.01	0.02 ± 0.01
C 22:5n-6	0.02 ± 0.01	0.03 ± 0.01
N-6	33.08 ± 4.94	50.12 ± 0.22
C 18:3n-3	3.77 ± 0.57	5.72 ± 0.05
C 20:3n-3	0.01 ± 0.01	0.01 ± 0.01
C 20:5n-3	0.14 ± 0.02	0.21 ± 0.01
C 22:5n-3	0.03 ± 0.01	0.05 ± 0.01
C 22:6n-3	0.15 ± 0.01	0.23 ± 0.05
N-3	4.10 ± 0.59	6.22 ± 0.09
Total	64.32 ± 9.24	97.54 ± 1.18

Values are mean ± SD from triplicate analysis. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 2. Fatty Acid Concentration of Gavage Oils

Fatty Acid	μg fatty acid/mg oil	
	Soybean Oil	DHASCO
C 10:0	0.29 ± 0.02	6.88 ± 1.16
C 12:0	0.23 ± 0.05	54.99 ± 1.40
C 14:0	1.06 ± 0.06	150.91 ± 2.97
C 16:0	123.03 ± 2.51	128.80 ± 3.65
C 17:0	1.07 ± 0.03	0.57 ± 0.08
C 18:0	48.18 ± 0.59	6.92 ± 0.15
C 20:0	3.61 ± 0.10	0.92 ± 0.10
C 22:0	3.04 ± 0.18	1.27 ± 0.06
C 23:0	0.33 ± 0.13	0.19 ± 0.04
C 24:0	1.12 ± 0.17	0.88 ± 0.64
SFAs	183.13 ± 2.80	353.08 ± 6.34
C 12:1	0.05 ± 0.04	0.12 ± 0.03
C 14:1	0.11 ± 0.06	3.48 ± 0.15
C 16:1	2.33 ± 0.21	31.10 ± 1.48
C 18:1n-7	19.93 ± 0.15	4.80 ± 0.56
C 18:1n-9	257.33 ± 3.56	186.10 ± 6.40
C 20:1n-9	1.69 ± 0.07	0.48 ± 0.03
C 22:1n-9	0.68 ± 0.06	0.57 ± 0.06
C 24:1n-9	0.13 ± 0.05	3.44 ± 1.10
MUFAs	282.43 ± 3.04	230.39 ± 7.90
C 18:2n-6	583.49 ± 17.75	6.53 ± 0.13
C 18:3n-6	0.11 ± 0.05	0.06 ± 0.01
C 20:2n-6	0.46 ± 0.03	0.06 ± 0.07
C 20:3n-6	0.08 ± 0.02	0.07 ± 0.02
C 20:4n-6	0.12 ± 0.13	0.09 ± 0.06
C 22:2n-6	0.13 ± 0.10	0.06 ± 0.04
C 22:4n-6	0.08 ± 0.05	0.05 ± 0.01
C 22:5n-6	0.05 ± 0.01	0.07 ± 0.03
N-6	584.52 ± 17.90	7.00 ± 0.21
C 18:3n-3	66.18 ± 2.90	0.61 ± 0.05
C 20:3n-3	0.09 ± 0.06	0.05 ± 0.01
C 20:5n-3	0.07 ± 0.07	0.19 ± 0.05
C 22:5n-3	0.06 ± 0.01	3.92 ± 0.17
C 22:6n-3	0.07 ± 0.01	337.50 ± 6.90
N-3	66.47 ± 2.93	342.27 ± 7.12
Total	1116.54 ± 23.59	932.75 ± 10.03

Values are mean \pm SD from triplicate analysis. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Chapter 5

Sex Differences in Response to Acute DHA Feeding in Sprague-Dawley Rats

5.1. Introduction

Several sex differences in cardiovascular physiology and fatty acid metabolism exist which may result in differential effects of acute DHA feeding in males and females. Females are less susceptible to ischemia-reperfusion injury (Bae & Zhang 2005), demonstrate increased resistance to myocardial oxidative stress (Lagranha et al. 2010), and have higher myocardial DHA content (Kitson et al. 2012) than males. Females are also less responsive to ischemic preconditioning induced-cardioprotection, which depends on an oxidative stress stimulus to initiate cardioprotective adaptations (Song et al. 2003; Turcato et al. 2006). Therefore, the effect of acute DHA intake on susceptibility to myocardial ischemia-reperfusion injury may be sex-dependent.

5.2. Study Protocols

Standard chow-fed male and female Sprague-Dawley rats (3 – 4 months of age, n = 10 per sex, diet composition detailed in Table 1) were gavaged with a single dose of oil normalized to body weight. The treatments were either algal-derived high DHA oil, providing 0.4 mg DHA/g body weight (0.339 mg DHA/ μ L oil), or an equivalent volume of soybean oil as a control. Following gavage, rats were returned to their cages and had *ad libitum* access to food and water for 12h and were then fasted for an additional 12h. Animals were then anaesthetized with intraperitoneal sodium pentobarbital, instrumented in the Langendorff isolated heart system, and perfused at a constant flow normalized to body weight. In this study a normalization period of 30 minutes was used, followed by 30 minutes of global ischemia and 90 minutes of reperfusion.

Left ventricular function and vascular performance parameters were measured continuously throughout the protocol. Hearts were then collected, frozen at -80°C, and analysed for infarction by TTC staining.

5.3. Results

Fourteen animals are included in the final analyses as 4 animals could not be instrumented due to technical difficulties with heart perfusion and 2 animals were excluded due to baseline left ventricular function that was below the *a priori* exclusion criteria. Therefore, the number of animals included in the analysis per group were: male control (3), male DHA (4), female control (4), female DHA (3). There was no effect of DHA treatment on animal weight ($p>0.05$), but males had significantly greater body weight than females ($p<0.0001$).

The ischemic stimulus used in these studies did not result in significant impairment of left ventricular function as compared with baseline values. In fact, recovery of hemodynamic function immediately following the ischemia-reperfusion protocol was actually higher than baseline (Figures 1 – 7).

The hearts of DHA-fed males had lower left ventricular function at baseline relative to male controls, but this affect was not observed in females (summarized in Table 3). Acute DHA feeding caused reduced left ventricular rate of contraction and rate of relaxation in male rats at baseline ($p<0.05$). There was also a trend towards reduced left ventricular developed pressure ($p=0.058$) and systolic pressure ($p=0.064$) although this did not reach statistical significance. There was no significant effect of DHA feeding at baseline on female heart function in any of the functional parameters measured.

Male control rats had significantly higher left ventricular function than female control animals, including increased developed pressure (Figure 1), rate of contraction (Figure 2), and

rate of relaxation (Figure 3) ($p < 0.05$). Treatment with DHA appeared to eliminate left ventricular functional differences between male and female rats, as the male DHA treated hearts demonstrated reduced contractile function. As a result, there were no significant differences in left ventricular function between DHA treated males and either control or DHA treated females at baseline ($p > 0.05$).

DHA-induced impairment of cardiac function persisted following ischemia into the reperfusion period, where DHA treatment was associated with approximately 25% reduced developed pressure compared with controls in males (Figure 1A). Rate of contraction (Figure 2A), rate of relaxation (Figure 3A), and systolic pressure (Figure 6A) during reperfusion were also lowered in the DHA treated male hearts compared with controls. By normalizing data to baseline values to eliminate the effect of baseline differences, it was determined that the percent recovery of heart contractile function was greater in the DHA treated hearts. Male hearts exhibited increased recovery of rate of contraction (Figure 2C), rate of relaxation (Figure 3C), and heart rate (Figure 4C). Therefore, despite lower absolute left ventricular function during reperfusion, the DHA treated animals actually recovered a greater percent of their baseline function following the ischemic stimulus. This protective effect of DHA on left ventricular function was only observed in males, as the percent recovery of contractile function in females was not affected.

There was no effect of DHA treatment or sex on coronary perfusion pressure (Figure 7). DHA also did not appear to have a protective effect on myocardial contracture. In fact, DHA actually appeared to worsen contracture, as hearts from male rats treated with DHA actually had significantly increased end diastolic pressure during and immediately following ischemia (Figure 5A, $p < 0.05$). There was no effect of DHA treatment on contracture in females (Figure 5B).

The effect of DHA on preservation of hemodynamic function in males, but not females, was not supported by infarction data as measured with TTC staining, as there was no effect of DHA treatment on infarction in male or female animals (Figure 8, $p>0.05$). However, when males and females were pooled together, there was a significant overall effect of DHA treatment on infarction independent of sex ($p<0.05$). There were additionally no significant differences in infarction damage between male and female Sprague-Dawley rats ($p>0.05$).

5.4. Discussion

These data suggest that the female response to DHA treatment differs markedly from males. Female hearts did not demonstrate increased recovery of any left ventricular functional parameters with DHA treatment. Baseline left ventricular functional differences with treatment, which were apparent in males, also did not occur in female hearts.

It was observed that DHA reduced baseline left ventricular function in males independent of the ischemia-reperfusion protocol. This impairment was observed in the rate of contraction and rate of relaxation, while there was also a trend towards reduced baseline developed pressure that did not reach significance. Baseline left ventricular function is dependent on numerous physiological systems, and the responses of the different functional variables measured in these studies provide some information as to the specific systems that are affected by DHA treatment. Rate of contraction is due to both the coupling of Ca^{2+} -induced Ca^{2+} release and the sensitivity of the myofilaments to Ca^{2+} . Myofilament Ca^{2+} sensitivity is reduced under conditions of oxidative stress (Suzuki et al. 1991). As DHA has been shown to increase cellular oxidative stress (Farina et al. 2003), it may have contributed to reduced left ventricular function through reductions in Ca^{2+} sensitivity.

The rate of relaxation is dependent on the removal of cytosolic Ca^{2+} , which proceeds mainly through 2 pathways in the murine myocardium, SERCA and NCX (Bassani et al. 1994). SERCA is the most dominant method for Ca^{2+} sequestration in rat cardiomyocytes, contributing ~92% of the Ca^{2+} sequestration capacity, with minimal contributions from the NCX and slow systems (Bers 2002; Bassani et al. 1994). SERCA function is impaired following ischemia-reperfusion injury (Temsah et al. 1999), as it is highly susceptible to oxidative stress and its function is reduced when specific sulfhydryl groups are oxidized (Scherer & Deamer 1986). Reduced SERCA activity could reduce the rate of removal of cytosolic Ca^{2+} , thus lowering the left ventricular rate of relaxation.

The developed pressure of the isolated heart is dependent on a combination of both the calcium sensitivity and the rate of relaxation, and since it takes into account both systolic and diastolic pressure it is indicative of contraction and relaxation mechanisms. Developed pressure could be reduced through a reduction in overall cytosolic Ca^{2+} during systole, reduced sequestration of cytosolic Ca^{2+} during diastole, or a reduction in myofilament Ca^{2+} sensitivity. As mentioned previously, oxidative stress can result in impairment in all 3 of these mechanisms, potentially resulting in depression of developed pressure in the isolated heart.

The effect of DHA treatment on left ventricular hemodynamics was sex-dependent, as no differences were seen in female rats. Females have higher innate levels of EPA and DHA in several tissues including the myocardium (Kitson et al. 2012). Female hearts additionally have increased oxidative stress handling capacity as compared with males, with increased myocardial levels of the enzymatic antioxidant SOD and reduced lipid peroxidation (Barp et al. 2002). Thus, if baseline functional differences were caused by increases in oxidative stress brought on

by DHA, this stressor may have been eliminated by the innately higher antioxidant defences in the female rats.

This study demonstrates that DHA decreases infarction damage following a single dose independent of sex. There was no significant effect in males and females when analyzed independently, but this may be due to reduced power due to low animal numbers. Evidence in the literature is mixed as to the role of DHA in reducing myocardial infarction, with some studies describing a reduction in myocardial infarction (Castillo et al. 2013; Zeghichi-Hamri et al. 2010) while others have reported no effect (Force et al. 1989). Protocol differences between these studies, including perfusion method and animal strain, may partially explain these contradictory results.

Previous reports in the literature have described increased infarction damage in males as compared with females (Edwards et al. 2009; Chicco et al. 2007; Bae & Zhang 2005). Conflictingly, the current study did not detect such a difference. This may be related to methodological factors, as there are a number of variables which differ between these studies, including animal species and strain, perfusion method used, and ischemic duration. Additionally, animal age may play some role, as sex-difference in cardiovascular function between males and females have been shown to dissipate with age. While there are significant differences in the response to ischemic preconditioning in 10 week old mice, these differences were no longer apparent at 18 weeks (Turcato et al. 2006).

Left ventricular function was higher than baseline following 30 minutes of global ischemia, indicating minimal hemodynamic impairment with the ischemic stimulus used. This observation conflicts with reports in the literature employing Sprague-Dawley rats with similar durations of global ischemia (Maddika et al. 2009; Bae & Zhang 2005), where significant impairment relative

to baseline was observed. This may be attributable to protocol differences between studies. One possibility is temperature regulation, as reduced temperature in the Langendorff preparation is known to be cardioprotective (Bell et al. 2011). Although water bath temperatures were controlled in these experiments, the temperature in the heart chamber and heart itself may have been lower than the target 37°C, resulting in reduced impairment from the ischemia-reperfusion protocol. The degree of functional impairment is also dependent on ischemic duration (Wang et al. 2001) and animal strain (Javouhey et al. 1989), and thus it may be possible to achieve demonstrable functional impairment by varying these parameters.

In conclusion, these results demonstrate a sex-dependent effect of DHA on left ventricular function at baseline and functional recovery following ischemia-reperfusion injury. They do not support sex differences in infarction which have previously been demonstrated in the literature, but do indicate a protective effect of DHA on infarction independent of sex. Protocol issues may have contributed to observations of greater left ventricular functional recovery following ischemia, and these should be investigated further to achieve demonstrable functional impairment.

Table 3. Baseline Effect of DHA Treatment in Sprague-Dawley Isolated Hearts				
	Male		Female	
	Control	DHA	Control	DHA
Developed Pressure	127.9 ± 5.9	80.0 ± 12.8	83.0 ± 6.1	65.8 ± 3.7
Systolic Pressure	133.8 ± 5.7	87.5 ± 13.0	88.1 ± 6.2	71.7 ± 2.5
Diastolic Pressure	5.9 ± 0.6	7.5 ± 0.8	5.1 ± 0.8	5.8 ± 1.6
Rate of Contraction	4519.4 ± 473.2	2809.0 ± 371.4*	2501.5 ± 244.6	2598.5 ± 248.0
Rate of Relaxation	2453.8 ± 135.7	1374.5 ± 212.5*	1520.5 ± 133.9	1233.7 ± 125.1
Heart Rate	242.8 ± 16.7	253.6 ± 13.5	214.7 ± 14.0	273.0 ± 18.3
Perfusion Pressure	60.5 ± 0.5	79.4 ± 16.2	113.6 ± 4.6	103.7 ± 8.8
Coronary Flow	15.5 ± 0.1	15.3 ± 0.2	12.3 ± 0.3	12.2 ± 0.1

* indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean ± S.E.M. (n=3 for male control and female DHA, n=4 for female control and male DHA).

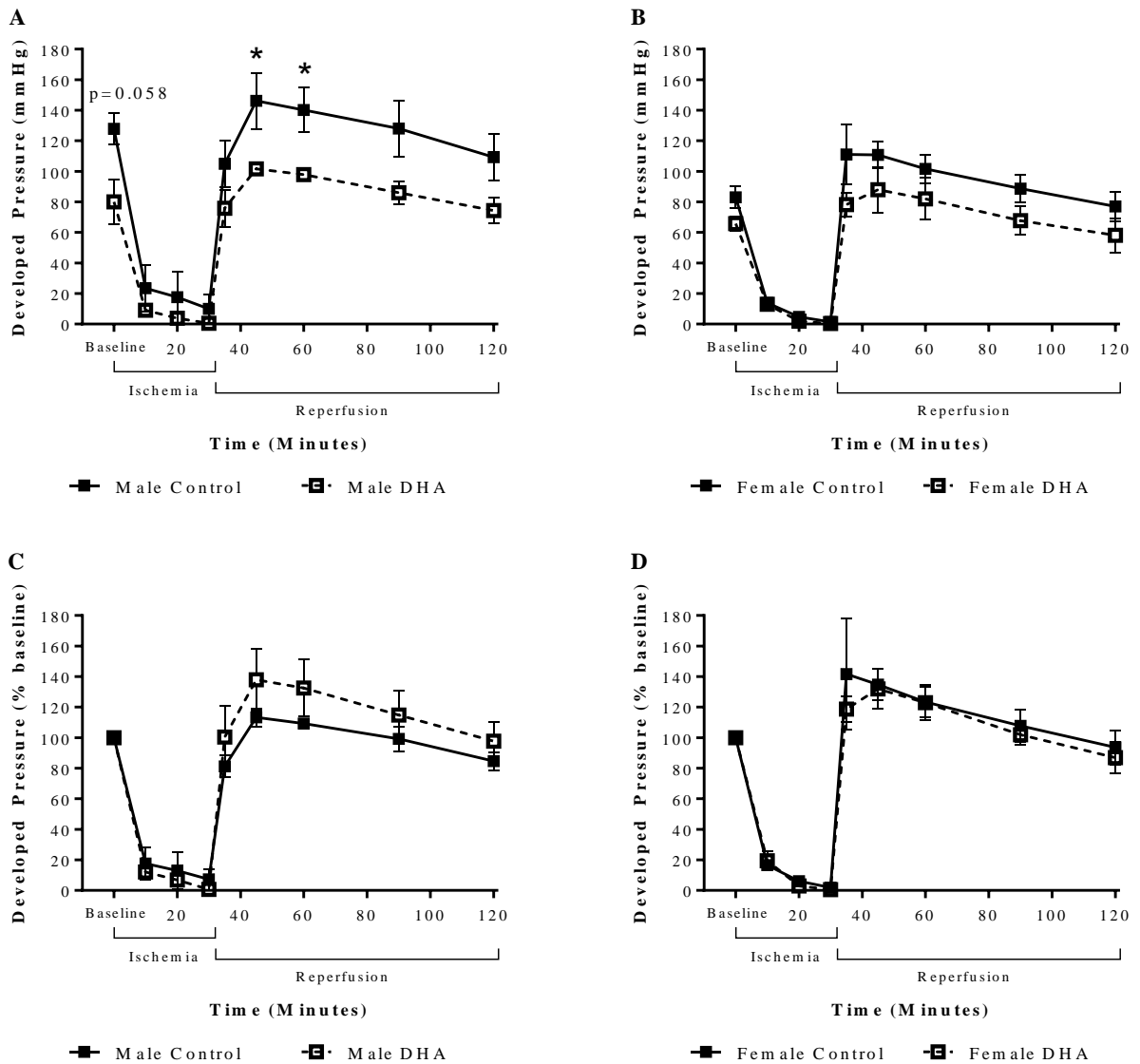


Figure 1. Left ventricular developed pressure in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Developed pressure is an index of the hearts ability to generate force and is calculated as the difference between systolic and diastolic pressure. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia.* indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

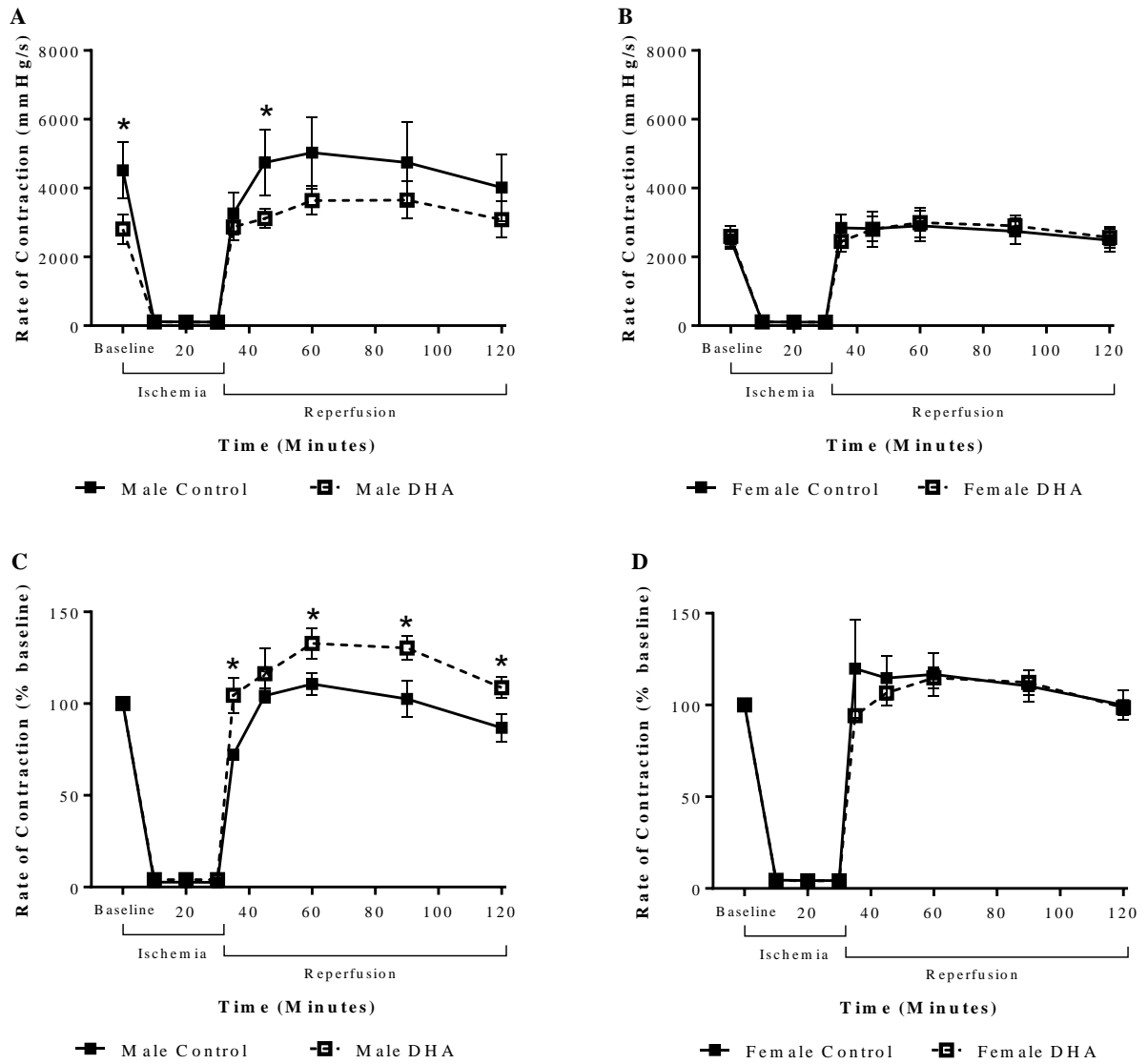


Figure 2. Left ventricular rate of contraction in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

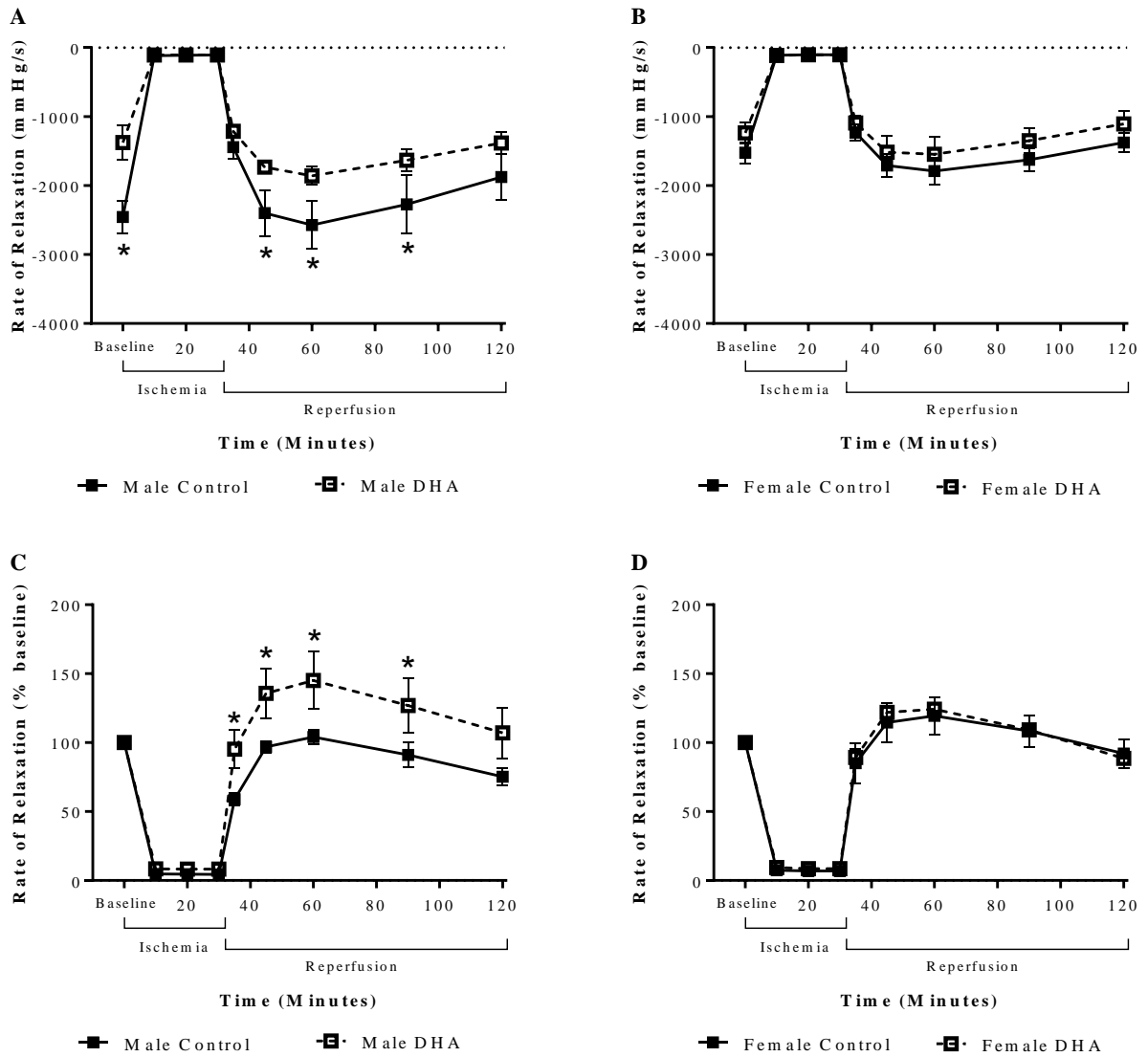


Figure 3. Left ventricular rate of relaxation in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

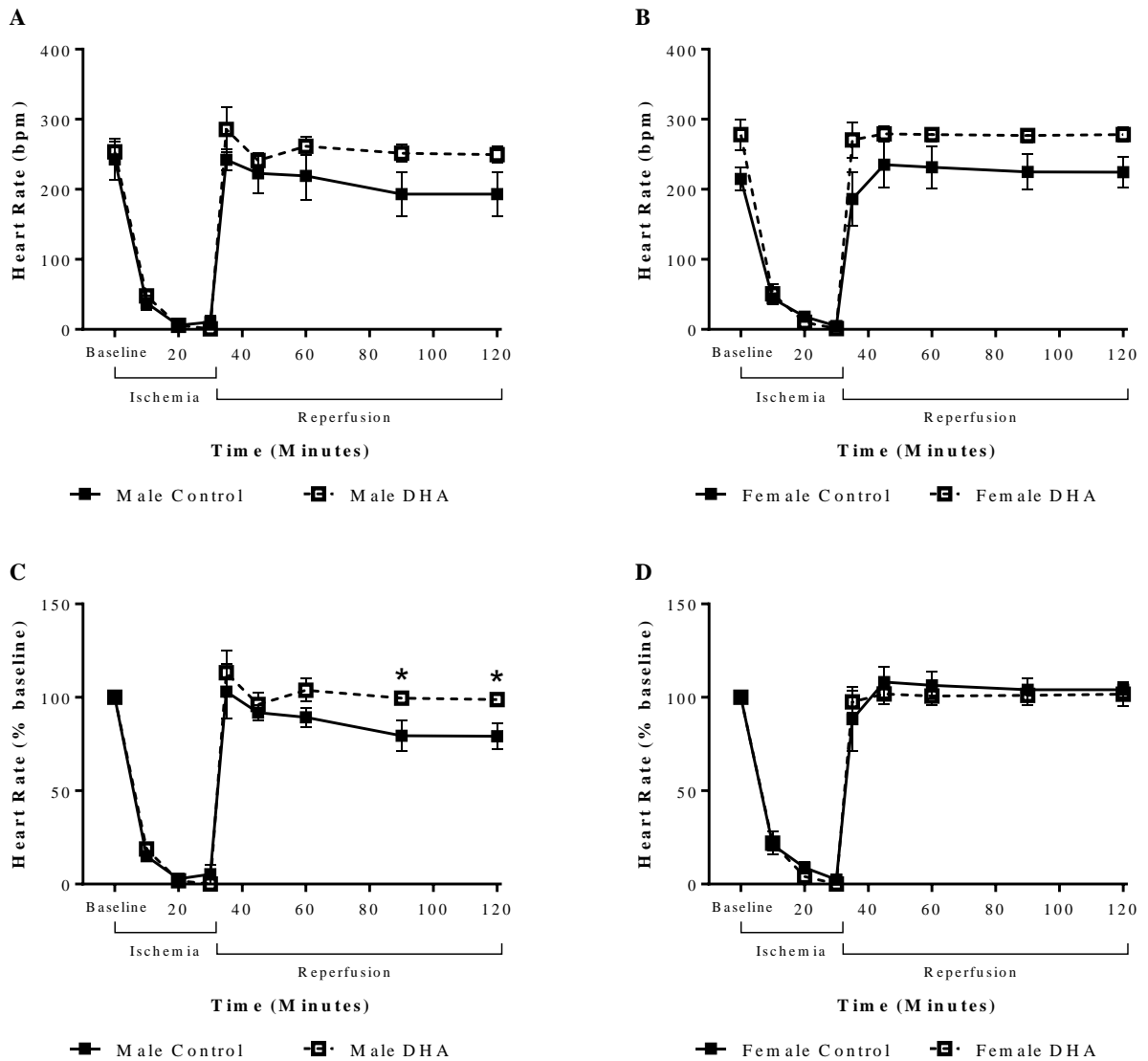


Figure 4. Heart rate of male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

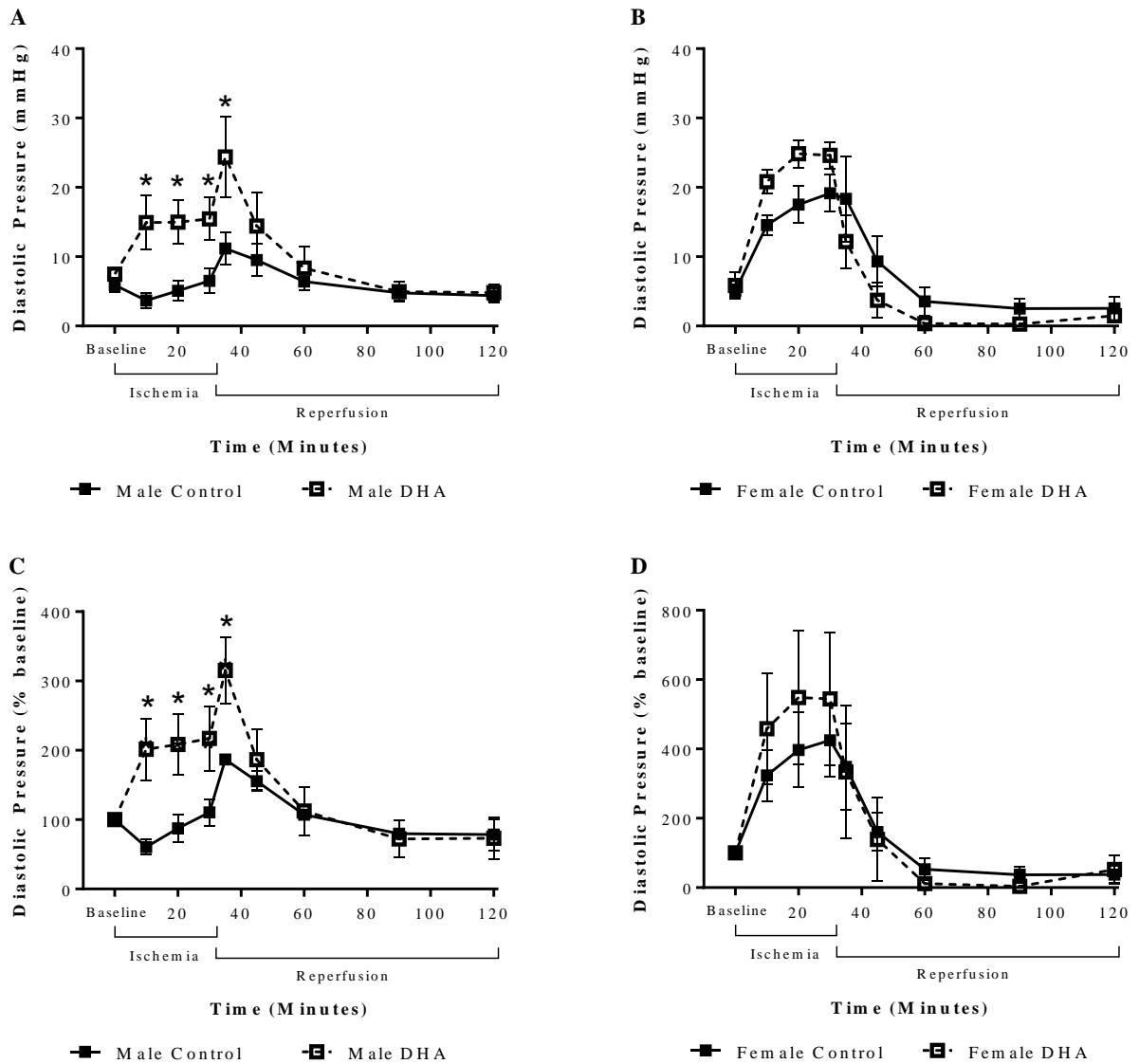


Figure 5. Left ventricular end diastolic pressure in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Diastolic pressure is an index of cardiac damage, with higher values being associated with increased myocardial damage following ischemia. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

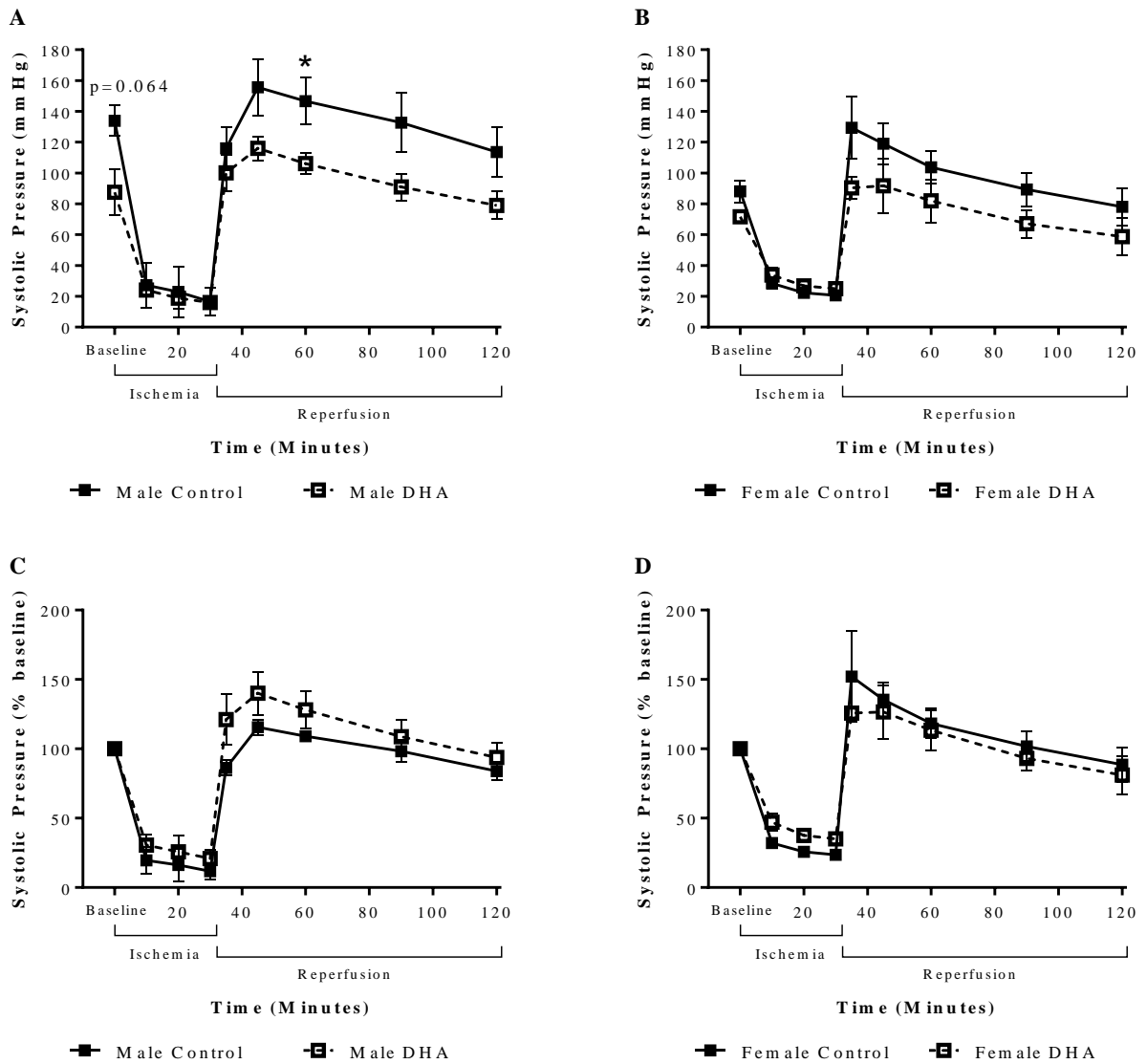


Figure 6. Left ventricular systolic pressure in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

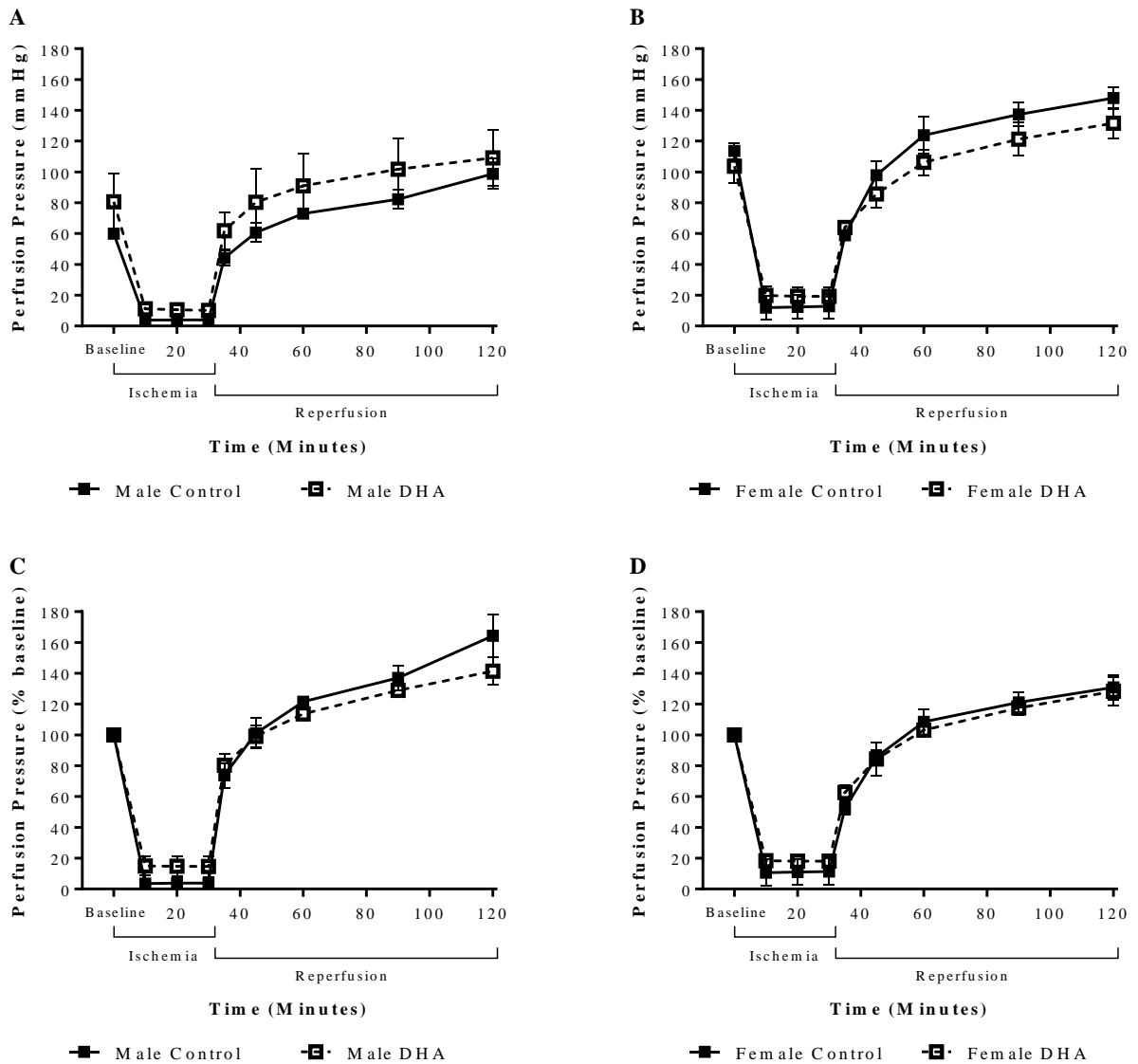


Figure 7. Systemic perfusion pressure in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Figures A and B denote absolute pressure data while Figures C and D are normalized to baseline and therefore indicate percent recovery following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=3$ for male control and female DHA, $n=4$ for female control and male DHA).

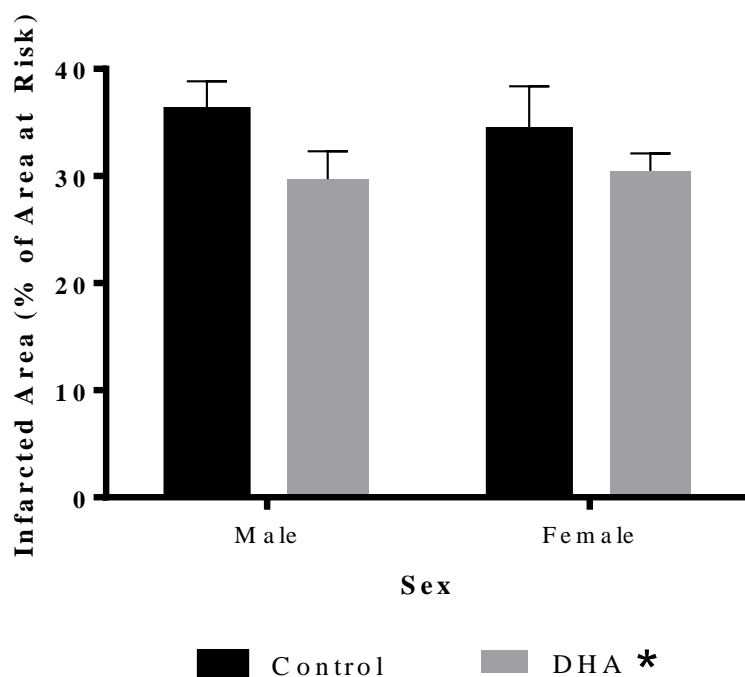


Figure 8. Infarcted area in male and female Sprague-Dawley rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates a significant effect of treatment by independent T-test of pooled values ($p < 0.05$). Values are expressed as mean \pm S.E.M. ($n=3$ for each male control and female DHA, $n=4$ for each female control and male DHA).

Chapter 6

Effect of Ischemic Duration on Ischemia-Reperfusion Injury in Sprague-Dawley Rats

6.1. Introduction

The duration of ischemia affects the degree of left ventricular functional impairment and myocardial infarction in isolated heart models (Wang et al. 2001). It was determined in the previous study that 30 minutes of ischemia permits recovery of left ventricular function above baseline values in the model used. Achieving demonstrable impairment following ischemia is essential in order to detect any changes in ischemic susceptibility from an experimental intervention. Therefore, it was necessary to investigate the effects of varying ischemia durations on myocardial recovery in Sprague-Dawley rats perfused in constant flow.

6.2. Study Protocols

Group housed male Sprague-Dawley rats (3 – 4 months of age, n = 9) who were maintained on the standard chow diet were sacrificed, instrumented in the Langendorff model, and perfused at constant flow proportional to body weight. Following a 30 minute baseline perfusion period, hearts were randomly assigned to one of the three groups: 20 minute, 30 minute, or 40 minute global ischemia. Following the ischemic stimulus, hearts were reperfused for a further 90 minutes. Left ventricular function and coronary vascular performance parameters were measured continuously throughout the protocol. Hearts were then collected, frozen at -80°C, and analysed for infarction by TTC staining.

6.3. Myocardial Response to Ischemia-Reperfusion

Three animals per group were included in the analyses. There were no differences in body weight between the ischemic duration groups ($p>0.05$). As hypothesized, progressive increases in ischemia duration resulted in corresponding decreases in left ventricular functional performance during the ischemia-reperfusion protocol. Prior to the ischemic stimulus, there were no significant baseline differences between any of the groups in any of the functional variables measured ($p>0.05$).

Forty minutes of ischemia resulted in significantly greater impairment of reperfusion developed pressure (Figure 9) and rate of contraction (Figure 10) as compared with 20 minutes of ischemia, and reperfusion end diastolic pressure (Figure 11) was significantly greater than both 20 and 30 minute groups. This effect was only significant in the first 15 minutes of the protocol, as after this point there were no group differences in left ventricular function with the exception of end diastolic pressure, which remained significantly elevated in the 40 minute group even after 120 minutes of reperfusion. These data suggest that ischemic impairment of left ventricular function typically manifests in the early phase of reperfusion in this model. Heart rate (Figure 12), vascular function (Figure 13), and systolic pressure (Figure 14) were not affected by the duration of ischemia, as these variables were not different at any point in the protocol ($p>0.05$). Ischemia duration also did not affect the cumulative measure of area under the curve (AUC) for any of these variables. Importantly, there no significant differences in either the left ventricular or coronary vascular function between the 20 minute and 30 minute groups, and therefore only the 40 minute group differed in the functional response to ischemia-reperfusion.

The area under the curve response was highly variable, resulting in difficulty in determining statistical significance. There was no effect of ischemia duration on the timecourse of rate of relaxation recovery (Figure 15). However, rate of relaxation demonstrated significant cumulative differences in the reperfusion period demonstrated by reduced AUC in the 40 minute relative to the 20 minute group.

Infarct size increased with increasing durations of ischemia (Figure 16). Infarction measurement by TTC staining did not appear to exactly replicate the trends observed in the left ventricular functional variables, as 20 and 30 minute groups were not significantly different in their functional impairment but exhibited significantly different infarct size. Therefore, the functional changes observed were not directly correlated with observations of structural changes in the myocardium.

6.4. Discussion

Ischemia duration was associated with increased functional impairment in some, but not all functional variables. Notably, developed pressure, rate of contraction, and rate of relaxation were highly susceptible to ischemic duration. Contracture, as measured by end diastolic pressure, was also significantly elevated with increased ischemic duration. There was no significant effect of ischemic duration on heart rate. Interestingly, increased left ventricular impairment with higher ischemic durations manifested in the early stages of reperfusion and these differences did not persist until the end of the protocol.

Left ventricular functional impairment can be caused by the increased oxidative stress associated with ischemia-reperfusion (Kim et al. 2006), as it impairs myofilament Ca^{2+} sensitivity (Suzuki et al. 1991) and ATPase-dependent Ca^{2+} sequestration (Temsah et al. 1999).

Oxidative stress also induces the mitochondrial permeability transition pore to open (Halestrap et al. 2004) resulting in apoptotic and necrotic cell death (Gottlieb et al. 1994; Reeve et al. 2007). Therefore, increased oxidative stress in the 40 minute group may have contributed to the observed impairments in left ventricular function.

Contracture is dependent on intracellular Ca^{2+} overload and a lack of ATP resulting in rigor-like contraction of the myofilaments (Piper et al. 2003) and is typically used as an indicator of ischemia-reperfusion injury. Increased contracture in the 40 minute ischemic group is indicative of increased intracellular Ca^{2+} accumulation as compared with the 20 and 30 minute group.

Coronary vascular function was also not affected by ischemia duration, as systemic perfusion pressure did not differ between groups. When using constant flow perfusion, the ability of the vasculature of the isolated heart to respond to ischemic conditions is removed. For this reason, constant pressure perfusion is preferred for studies of ischemia-reperfusion injury (Skrzypiec-Spring et al. 2006).

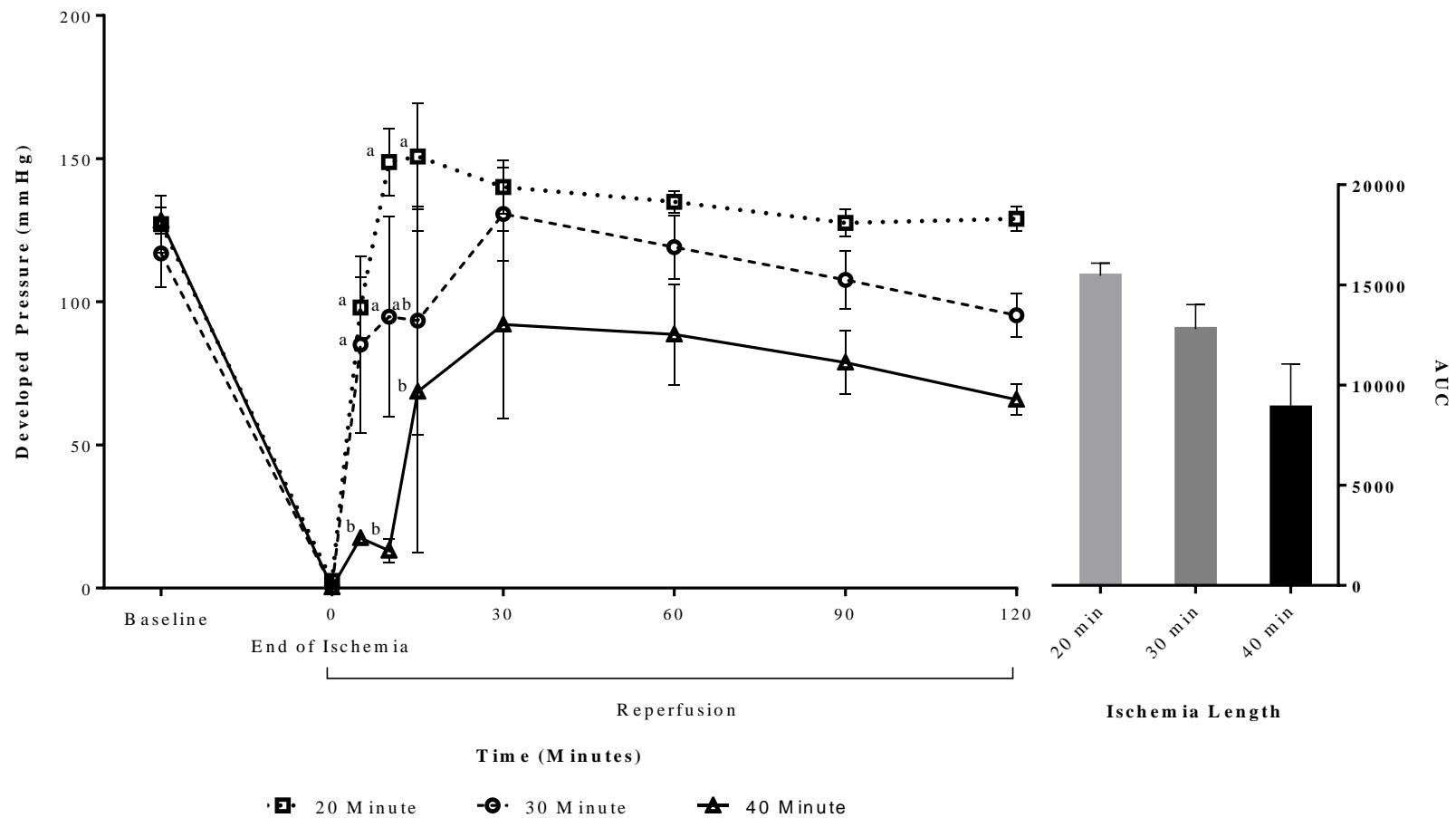


Figure 9. The effect of ischemia duration on left ventricular developed pressure. Developed pressure is an index of the hearts ability to generate force and is calculated as the difference between systolic and diastolic pressure. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual time, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n=3$ per group).

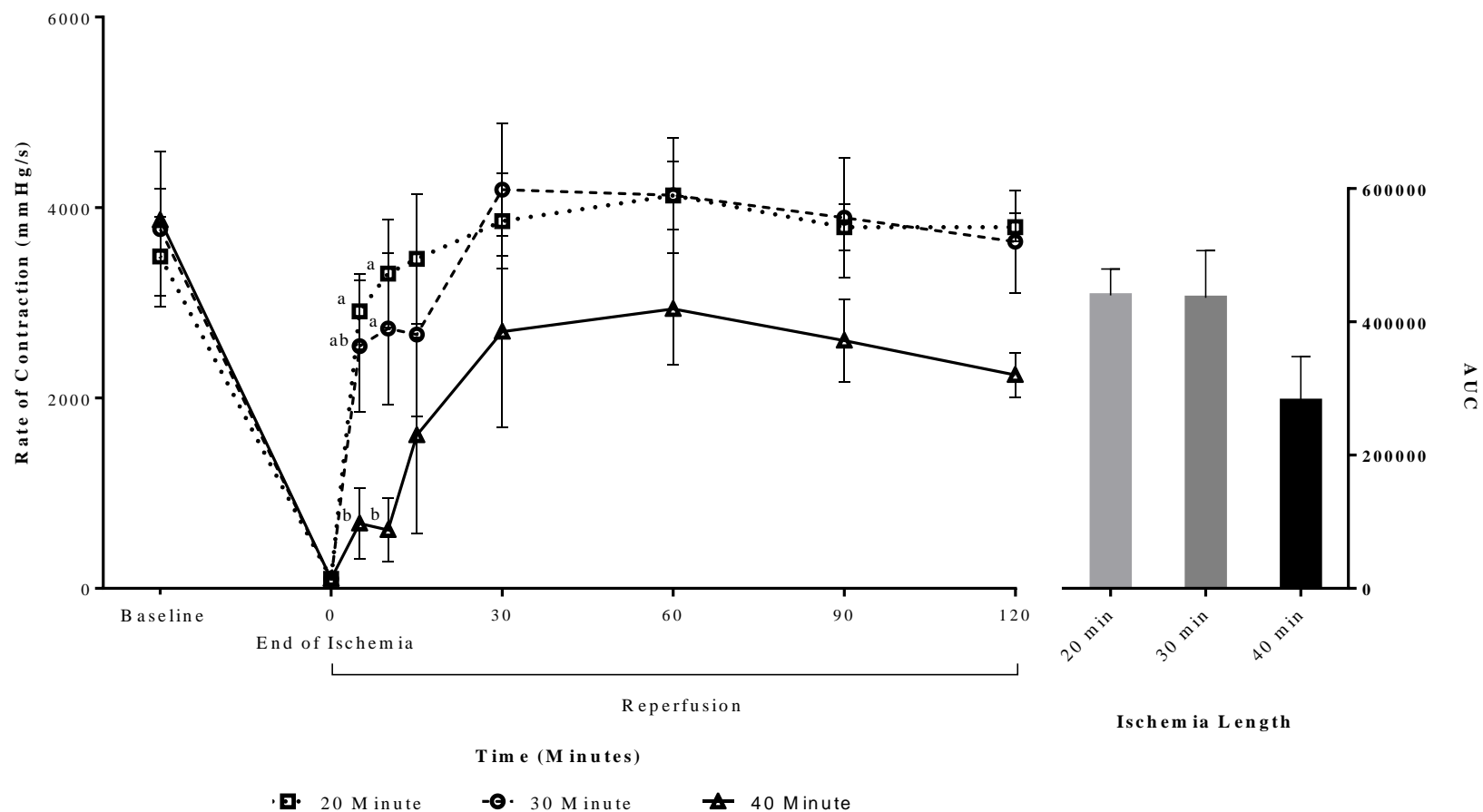


Figure 10. The effect of ischemia duration on left ventricular rate of contraction. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n=3$ per group).

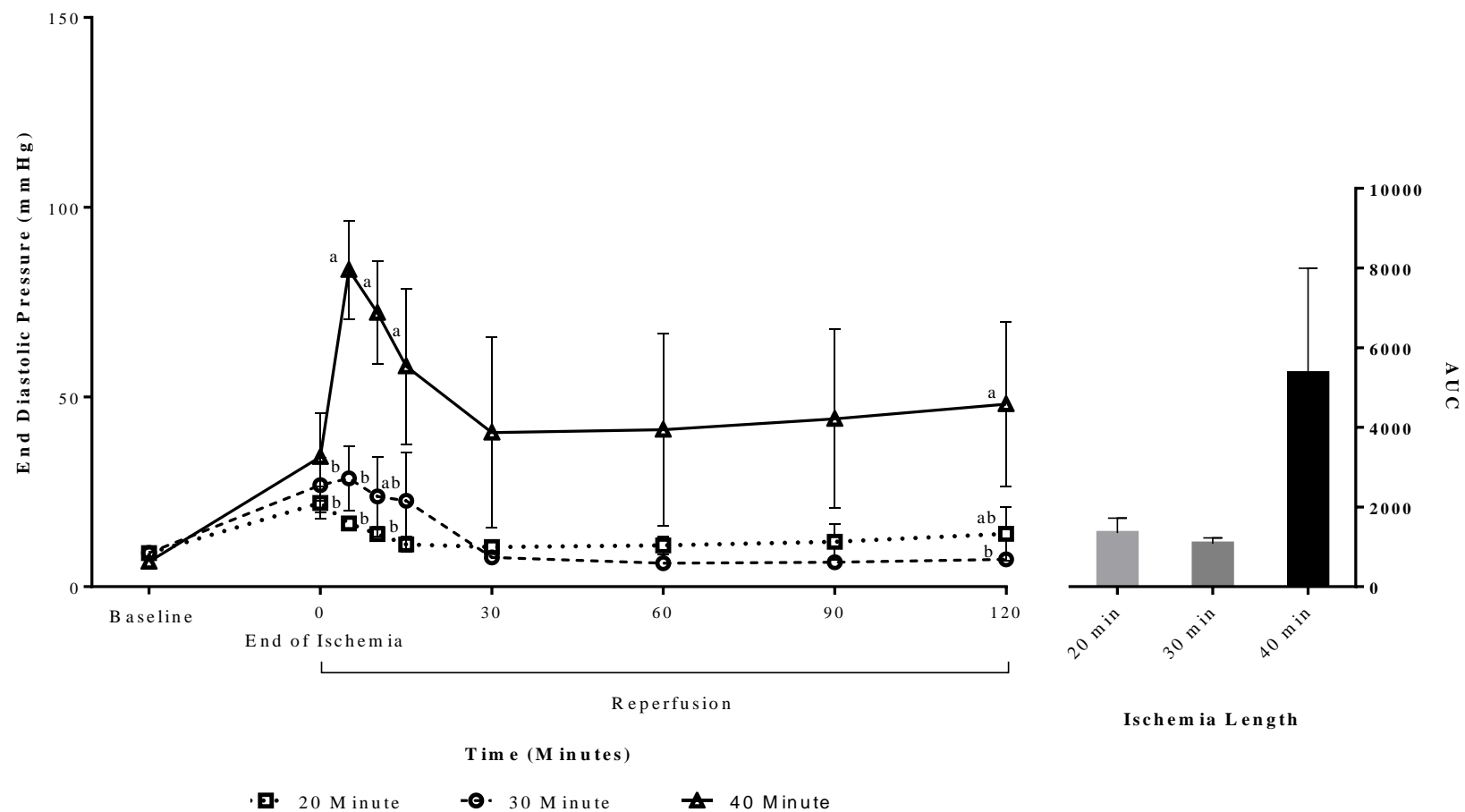


Figure 11. The effect of ischemia duration on left ventricular end diastolic pressure. Diastolic pressure is an index of cardiac damage, with higher values being associated with increased myocardial damage following ischemia. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n = 3$ per group).

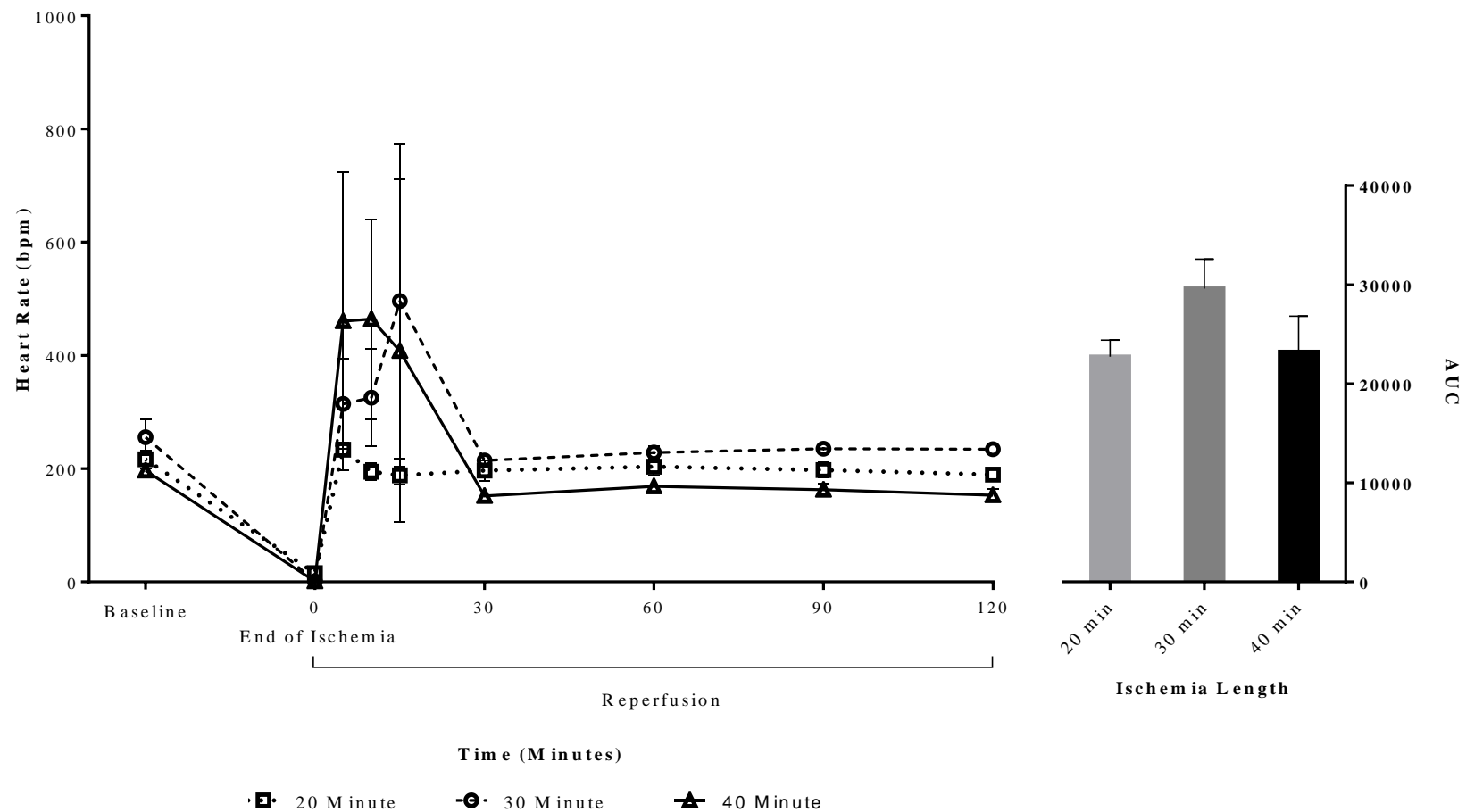


Figure 12. The effect of ischemia duration on heart rate. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n = 3$ per group).

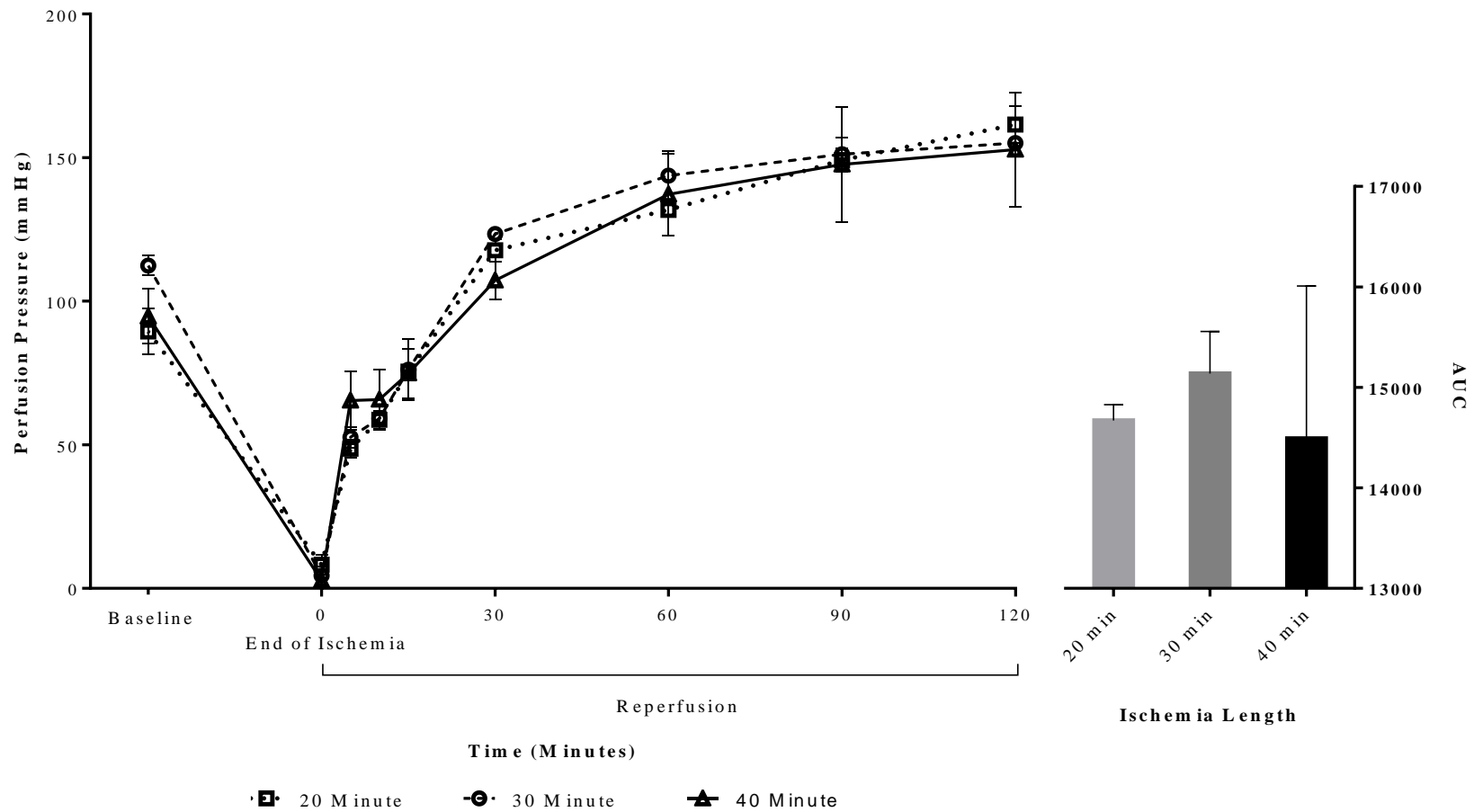


Figure 13. The effect of ischemia duration on systemic perfusion pressure. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M ($n=3$ per group).

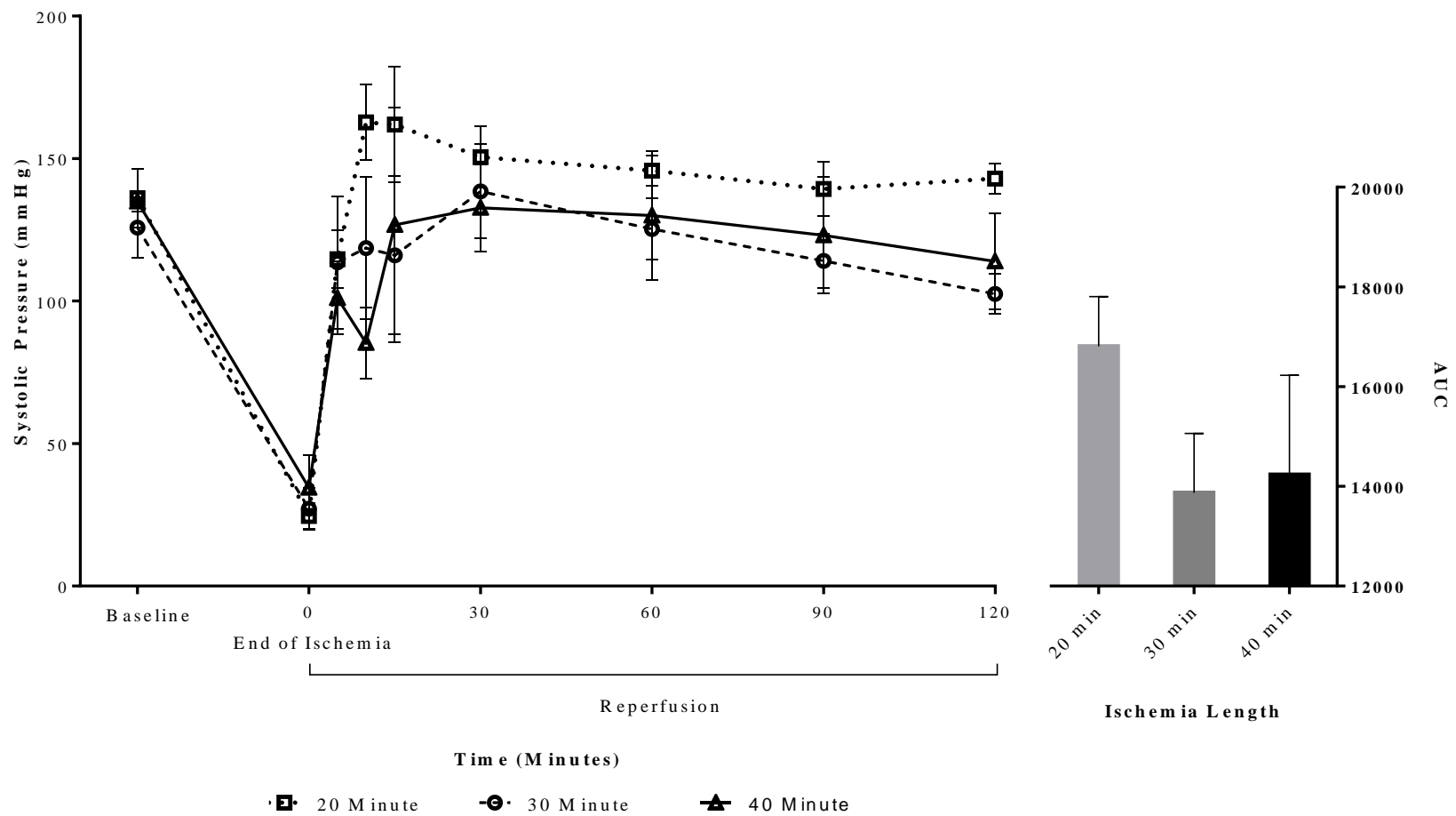


Figure 14. The effect of ischemia duration on left ventricular systolic pressure. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n=3$ per group).

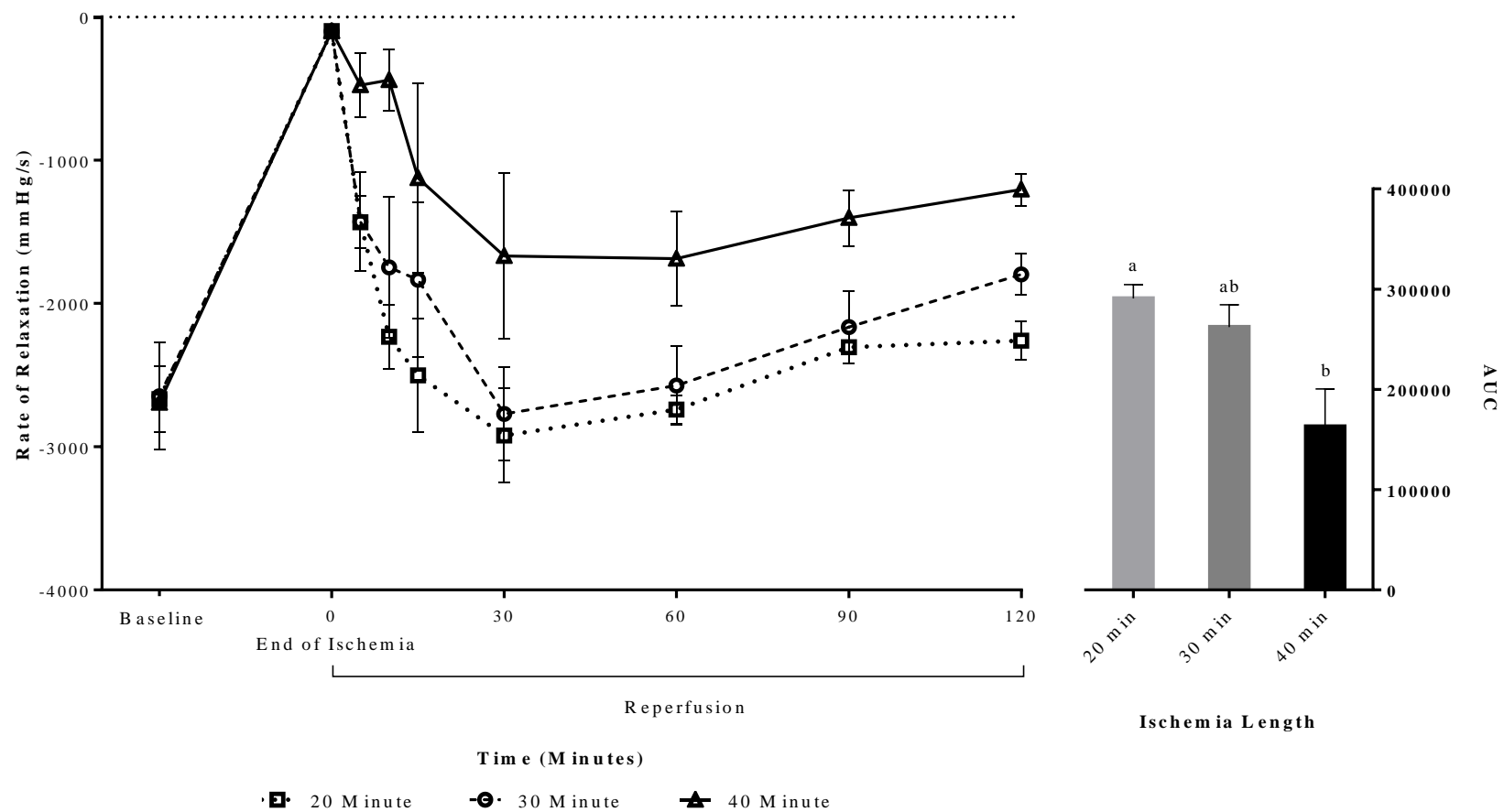


Figure 15. The effect of ischemia duration on left ventricular rate of relaxation. Curves were analyzed by 2 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. At each individual timepoint, points with different letters are significantly different ($p < 0.05$). AUC data was analyzed by 1 way ANOVA. Values are expressed as mean \pm S.E.M. ($n=3$ per group).

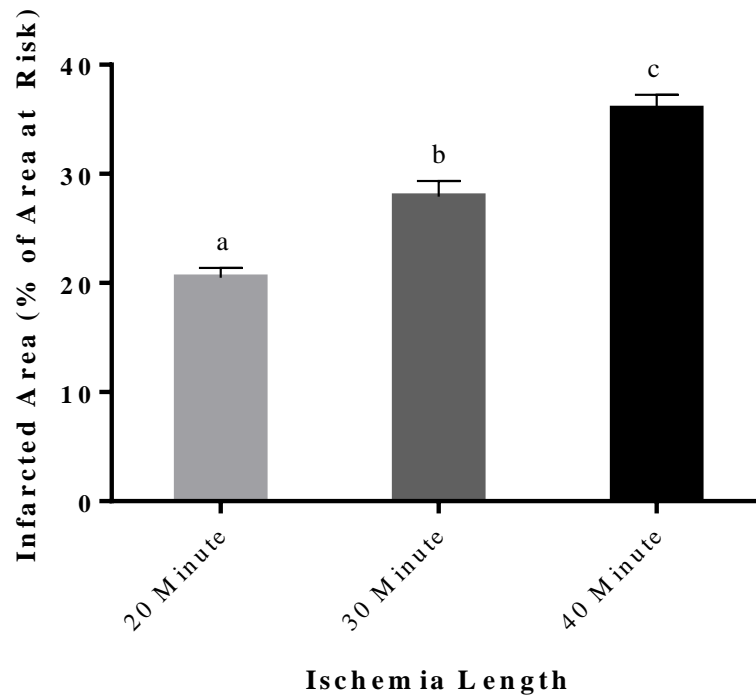


Figure 16. The effect of increasing ischemia duration on infarcted area in male Sprague-Dawley rats. Data was analyzed by 1 way ANOVA with Tukey's multiple comparison test following a significant interaction effect. Points with different letters are significantly different ($p < 0.05$). Values are expressed as mean \pm S.E.M.

Chapter 7

Effect of Acute DHA Feeding on Ischemia-Reperfusion Injury in Male Wistar Rats

7.1. Introduction

Wistar rats are more susceptible to ischemia-reperfusion injury as compared with Sprague-Dawley rats (Javouhey et al. 1989). Additionally, constant pressure perfusion is a more physiologically relevant model and is preferred to constant flow perfusion for studies of ischemia-reperfusion injury as it permits the normal autoregulatory function of the coronary vasculature (Skrzypiec-Spring et al. 2006). Previous investigations demonstrated that left ventricular functional recovery from ischemia-reperfusion of male rats is improved relative to controls with a single DHA feeding after 24 hours, while there is no effect in females. Therefore, the effect of a single DHA feeding on ischemic susceptibility was investigated utilizing male Wistar rats in constant pressure perfusion.

7.2. Study Protocols

Standard chow fed male Wistar rats (3 – 4 months of age, n = 12) were gavaged with a single dose of oil normalized to body weight. The treatments were either algal-derived high DHA oil providing 0.4 mg DHA/g body weight (0.339 mg DHA/ μ L oil) or an equivalent volume of soybean oil as a control. Following gavage, rats were returned to their cages and had ad libitum access to food and water for 12h and were then fasted for an additional 12h. Animals were anaesthetized at 24 hours with intraperitoneal sodium pentobarbital. Plasma samples were collected prior to heart excision by drawing blood from the inferior vena cava using a needle and syringe. Collected blood samples were then centrifuged at 3000 rpm for 10 minutes and the plasma layer was extracted and frozen at -80°C. Total fatty acids were determined by direct

methylation as described in section 3.4. Hearts were instrumented in the Langendorff isolated heart system and perfused at a constant pressure of 70 mmHg. A normalization period of 30 minutes was used, followed by 20 minutes of global ischemia and 120 minutes of reperfusion. Left ventricular function and vascular performance parameters were measured continuously throughout the protocol. Hearts were then collected, frozen at -80°C, and analysed for infarction by TTC staining (Chapter 3).

7.3. Myocardial Response to Ischemia-Reperfusion

Two hearts, one each from the control and DHA group, were excluded from further analysis at baseline due to heart rates below the *a priori* exclusion criteria (<175 bpm). Therefore 5 animals each are included in the analyses for both the DHA and control groups. There were no significant differences in body weight between DHA-treated and control animals ($p < 0.05$).

At 24 hours, the DHA treatment did not result in the same functional impairment that was observed in the Sprague-Dawley model. There were no significant baseline differences between DHA treatment and control in any of the functional variables measured ($p > 0.05$).

DHA was associated with improved left ventricular function following the ischemic stimulus. During reperfusion, the left ventricular rate of contraction (Figure 17) was significantly higher in DHA treated rats as compared with controls. There was a trend towards increasing reperfusion developed pressure in DHA treated rats, but this failed to reach significance (Figure 18). DHA also affected the function of the coronary vasculature, as coronary flow during reperfusion was greater in DHA treated hearts as compared with controls (Figure 19). There was no effect of DHA treatment on myocardial contracture as measured by diastolic pressure ($p > 0.05$, Figure 20), or the rate of relaxation (Figure 21), systolic pressure (Figure 22), or heart rate (Figure 23).

When data was normalized to baseline (data not shown), there was no significant effect of DHA treatment on any of the functional variables measured. There was no effect of DHA treatment on infarct size as measured by TTC staining ($p>0.05$, Figure 24).

7.4. Plasma Fatty Acids

Plasma n-3 HUFA were significantly elevated in the DHA group relative to control 24 hours following a single oral gavage in male Wistar rats (Tables 4 and 5). Plasma DHA concentration ($p=0.003$) was significantly increased in DHA treated animals relative to controls following oral gavage. Interestingly, plasma EPA significantly increased in concentration ($p=0.013$), even though the DHA gavage contains only trace amounts of EPA, suggesting the possibility of DHA retro-conversion to EPA. The increase in plasma n-3 PUFAs was associated with a corresponding decrease in plasma n-6 PUFAs. Arachidonic acid relative percent ($p=0.025$) but not absolute concentration ($p=0.466$) was reduced. Similarly, adrenic acid (22:4n-6) relative percent ($p=0.033$) but not absolute concentration ($p=0.151$) was reduced. The DHA oil also contains high levels of saturated fatty acids (Table 1) which did not appear to affect the plasma fatty acid composition. In fact, the only difference in saturated fatty acids between control and DHA treatment was the decrease of the concentration of saturated fatty acid 22:0, behenic acid ($p<0.05$).

7.5. Discussion

DHA treatment increased plasma EPA and DHA levels after 24 hours. This is in agreement with human studies that have demonstrated that plasma DHA peaks between 4 (Hanwell et al. 2009) and 6 hours (Harris et al. 2013) after a single n-3 HUFA intake, with levels declining, but remaining elevated relative to baseline, for at least 24 hours (Yang et al. 2012).

Increased plasma levels of EPA and DHA may impact myocardial function through modulation of ion channel kinetics. DHA interacts with, and can alter the kinetics, of the voltage gated L-type Ca^{2+} channel (Xiao et al. 1997) and the alpha subunit of the sodium channel (Xiao et al. 2001). Isolated heart studies have demonstrated myocardial protection from ischemia-reperfusion injury with infusion of n-3 HUFA directly into the perfusate likely demonstrating an effect of DHA in the free fatty acid pool (Smith et al. 2012; Richard et al. 2014). DHA as a free fatty acid affected the antioxidant capacity of the myocardium by reducing lipid peroxides and increased expression of Notch2, part of a signalling pathway that regulates cell fate (Richard et al. 2014). Increased plasma DHA can also be incorporated into tissues including the myocardium, where it may exert cardioprotective effects through modulation of one of the various mechanisms through which DHA is thought to confer cardioprotection including membrane structure and function, lipid mediator signalling, or oxidative-stress dependent preconditioning.

DHA treatment improved left ventricular function during reperfusion in isolated hearts from male Wistar rats perfused at constant pressure. The literature is mixed on whether DHA can improve hemodynamics following ischemia-reperfusion. Left ventricular function recovery from myocardial ischemia-reperfusion was not improved following 72 hours of n-3 HUFA infusion (McGuinness et al. 2006) and >6 weeks of n-3 HUFA feeding (Force et al. 1989; Zeghichi-Hamri et al. 2010). However, other studies have demonstrated improved left ventricular function following both traditional ischemic preconditioning stimuli (Abdukeyum et al. 2008) and chronic dietary intervention with n-3 HUFA (Castillo et al. 2013; Abdukeyum et al. 2008).

It was observed that acute DHA increased post-ischemic coronary flow, but this only occurred in the late stages of reperfusion, and was not evident when data was normalized to

baseline. DHA has previously been shown to affect vascular function following ischemia-reperfusion, as it improves the post-ischemic functioning of the microvasculature in the perfused hamster cheek pouch preparation (de Souza et al. 2015). Dietary intake of n-3 HUFA also increased coronary flow following *in vivo* ischemia-reperfusion injury in the rat (Force et al. 1989). There was no effect of DHA treatment on the response of the coronary vasculature immediately following ischemia, indicating that DHA treatment did not affect the reactive hyperemic response, which is the transient increase in coronary flow following ischemia-reperfusion that is caused by a build-up of vasodilatory metabolites (Khanamiri et al. 2013) which may actually increase the degree of myocardial damage during reperfusion (Olivecrona et al. 2007).

DHA treatment in this experiment did not reduce myocardial infarction area as measured by TTC staining. This is in contrast to the previous observation in Sprague Dawley rats using a constant flow protocol, although is unclear if this is due to rat strain differences or the impact of constant flow vs. constant pressure protocols. It may be possible that the effect of DHA treatment may not overcome the increased sensitivity of Wistar rats to ischemia-reperfusion injury (Javouhey et al. 1989), resulting in no observed differences in infarction. In addition, the constant pressure protocol results in a different cardiac challenge. Constant pressure, as opposed to constant flow perfusion, more closely approximates *in vivo* reperfusion, and a study employing constant pressure perfusion in ischemia-reperfusion models has shown no effect on infarction with n-3 HUFA feeding (Force et al. 1989). The protective effect of DHA on infarction injury may then be partly dependent on a constant flow protocol, and thus was not observed in this study employing constant pressure perfusion. In conclusion, in male Wistar rats there is evidence of preservation of myocardial function with DHA treatment, with increased left

ventricular rate of contraction and coronary flow in the reperfusion period. However, in this model these changes were not associated with reductions in infarction as previously observed in Sprague-Dawley rats.

Table 4. Effect of Gavage on Plasma Fatty Acid Weight Percent

Fatty Acid	Weight Percent (g/100g fatty acids)	
	Control	DHA
C 10:0	0.12 ± 0.07	0.12 ± 0.05
C 12:0	0.04 ± 0.03	0.05 ± 0.03
C 14:0	0.25 ± 0.04	0.28 ± 0.04
C 16:0	18.76 ± 0.94	18.91 ± 0.77
C 17:0	0.51 ± 0.05	0.51 ± 0.05
C 18:0	15.17 ± 1.20	14.38 ± 1.56
C 20:0	0.19 ± 0.04	0.16 ± 0.04
C 22:0	0.38 ± 0.07	0.31 ± 0.06*
C 23:0	0.29 ± 0.07	0.25 ± 0.06
C 24:0	0.80 ± 0.15	0.74 ± 0.14
Total SFA	36.85 ± 0.99	36.05 ± 1.45
C 12:1	0.02 ± 0.01	0.03 ± 0.02
C 14:1	0.03 ± 0.03	0.03 ± 0.03
C 16:1	0.63 ± 0.19	0.65 ± 0.17
C 18:1n-7	1.90 ± 0.22	1.82 ± 0.19
C 18:1n-9	6.28 ± 0.89	7.08 ± 0.94
C 20:1n-9	0.13 ± 0.02	0.12 ± 0.03
C 22:1n-9	0.13 ± 0.07	0.14 ± 0.07
C 24:1n-9	0.71 ± 0.09	0.69 ± 0.14
Total MUFA	9.85 ± 0.98	10.58 ± 1.06
C 18:2n-6	19.47 ± 1.32	20.51 ± 2.13
C 18:3n-6	0.29 ± 0.06	0.26 ± 0.07
C 20:2n-6	0.32 ± 0.06	0.28 ± 0.05
C 20:3n-6	0.42 ± 0.09	0.46 ± 0.09
C 20:4n-6	26.93 ± 1.59	24.65 ± 2.48*
C 22:2n-6	0.14 ± 0.06	0.13 ± 0.03
C 22:4n-6	0.34 ± 0.07	0.27 ± 0.05*
C 22:5n-6	0.18 ± 0.08	0.19 ± 0.06
Total n-6 PUFA	48.09 ± 1.23	46.76 ± 1.86
C 18:3n-3	0.59 ± 0.11	0.62 ± 0.21
C 20:3n-3	0.06 ± 0.03	0.06 ± 0.03
C 20:5n-3	0.54 ± 0.11	0.82 ± 0.15*
C 22:5n-3	0.57 ± 0.10	0.52 ± 0.08
C 22:6n-3	2.10 ± 0.18	3.15 ± 0.34*
Total N-3 PUFA	3.85 ± 0.31	5.17 ± 0.55*
Total Fatty Acids	98.62 ± 0.26	98.56 ± 0.39

Data is expressed as mean ± SD (n = 10 per group). * indicates significantly different from controls (p < 0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 5. Effect of Gavage on Plasma Total Fatty Acid Concentration

Fatty Acid	μg fatty acid/100 mL plasma	
	Control	DHA
C 10:0	0.12 ± 0.06	0.14 ± 0.07
C 12:0	0.04 ± 0.03	0.05 ± 0.03
C 14:0	0.27 ± 0.05	0.30 ± 0.09
C 16:0	19.74 ± 3.21	20.70 ± 4.60
C 17:0	0.53 ± 0.07	0.55 ± 0.09
C 18:0	15.96 ± 2.75	15.76 ± 3.86
C 20:0	0.19 ± 0.04	0.18 ± 0.04
C 22:0	0.39 ± 0.05	$0.33 \pm 0.07^*$
C 23:0	0.30 ± 0.06	0.27 ± 0.07
C 24:0	0.83 ± 0.09	0.79 ± 0.13
Total SFA	38.72 ± 5.61	39.44 ± 8.49
C 12:1	0.02 ± 0.01	0.03 ± 0.02
C 14:1	0.03 ± 0.03	0.04 ± 0.03
C 16:1	0.66 ± 0.20	0.74 ± 0.41
C 18:1n-7	2.01 ± 0.49	2.02 ± 0.63
C 18:1n-9	6.63 ± 1.52	7.80 ± 2.31
C 20:1n-9	0.13 ± 0.02	0.13 ± 0.03
C 22:1n-9	0.13 ± 0.07	0.15 ± 0.08
C 24:1n-9	0.74 ± 0.06	0.74 ± 0.13
Total MUFA	10.38 ± 2.06	11.67 ± 3.36
C 18:2n-6	20.48 ± 3.36	22.53 ± 5.86
C 18:3n-6	0.31 ± 0.10	0.30 ± 0.16
C 20:2n-6	0.33 ± 0.05	0.30 ± 0.09
C 20:3n-6	0.44 ± 0.11	0.51 ± 0.18
C 20:4n-6	28.50 ± 5.69	26.74 ± 4.85
C 22:2n-6	0.14 ± 0.05	0.14 ± 0.03
C 22:4n-6	0.35 ± 0.05	0.30 ± 0.09
C 22:5n-6	0.19 ± 0.10	0.22 ± 0.09
Total n-6 PUFA	50.73 ± 8.85	51.03 ± 10.36
C 18:3n-3	0.62 ± 0.15	0.70 ± 0.37
C 20:3n-3	0.06 ± 0.03	0.07 ± 0.04
C 20:5n-3	0.58 ± 0.19	$0.91 \pm 0.33^*$
C 22:5n-3	0.60 ± 0.14	0.58 ± 0.22
C 22:6n-3	2.23 ± 0.53	$3.49 \pm 1.02^*$
Total N-3 PUFA	4.08 ± 0.90	$5.75 \pm 1.85^*$
Total Fatty Acids	103.91 ± 16.94	107.89 ± 23.55

Data is expressed as mean \pm SD (n = 10 per group) * indicates significantly different from controls (p < 0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

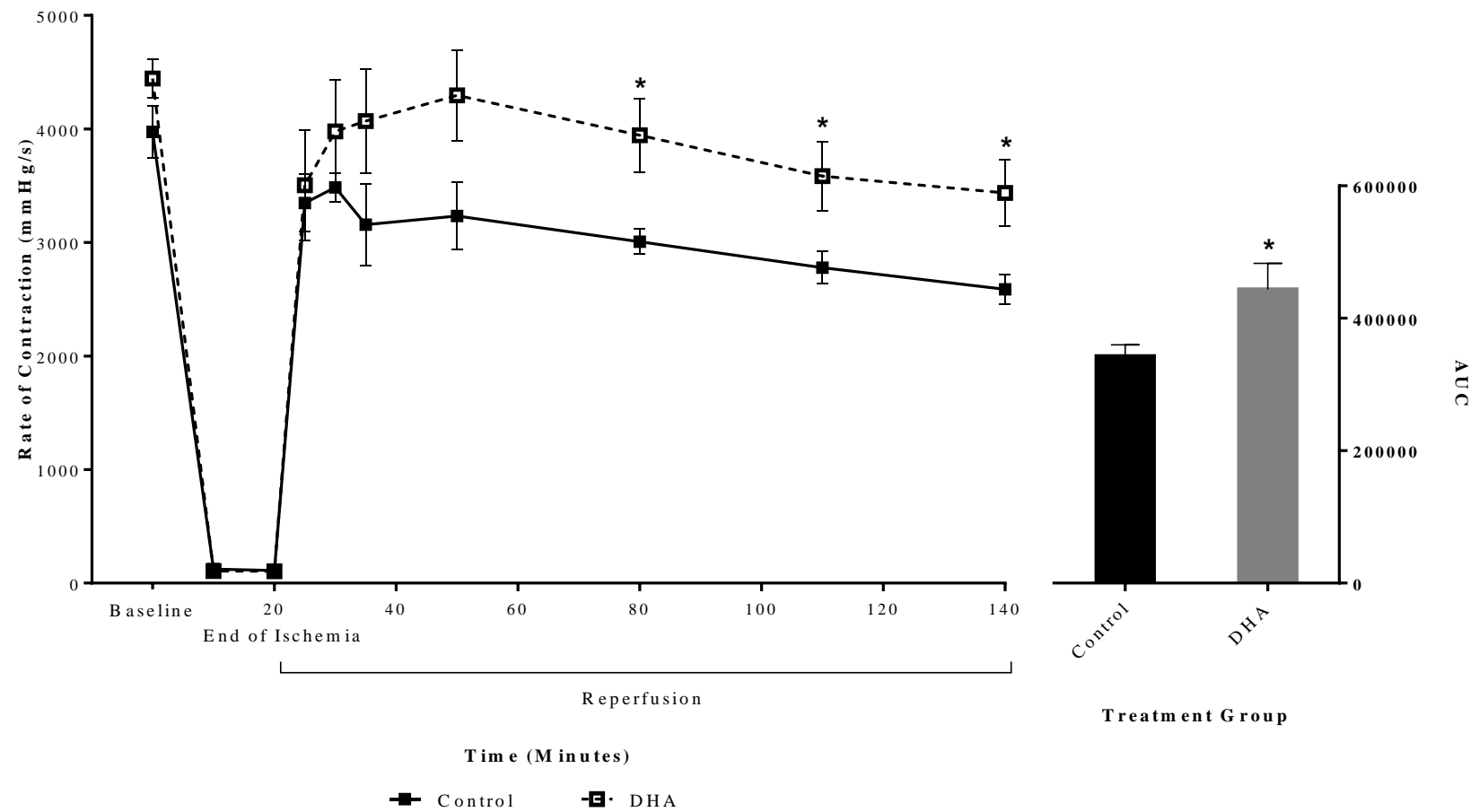


Figure 17. Left ventricular rate of contraction in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates significantly different ($p<0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=5$).

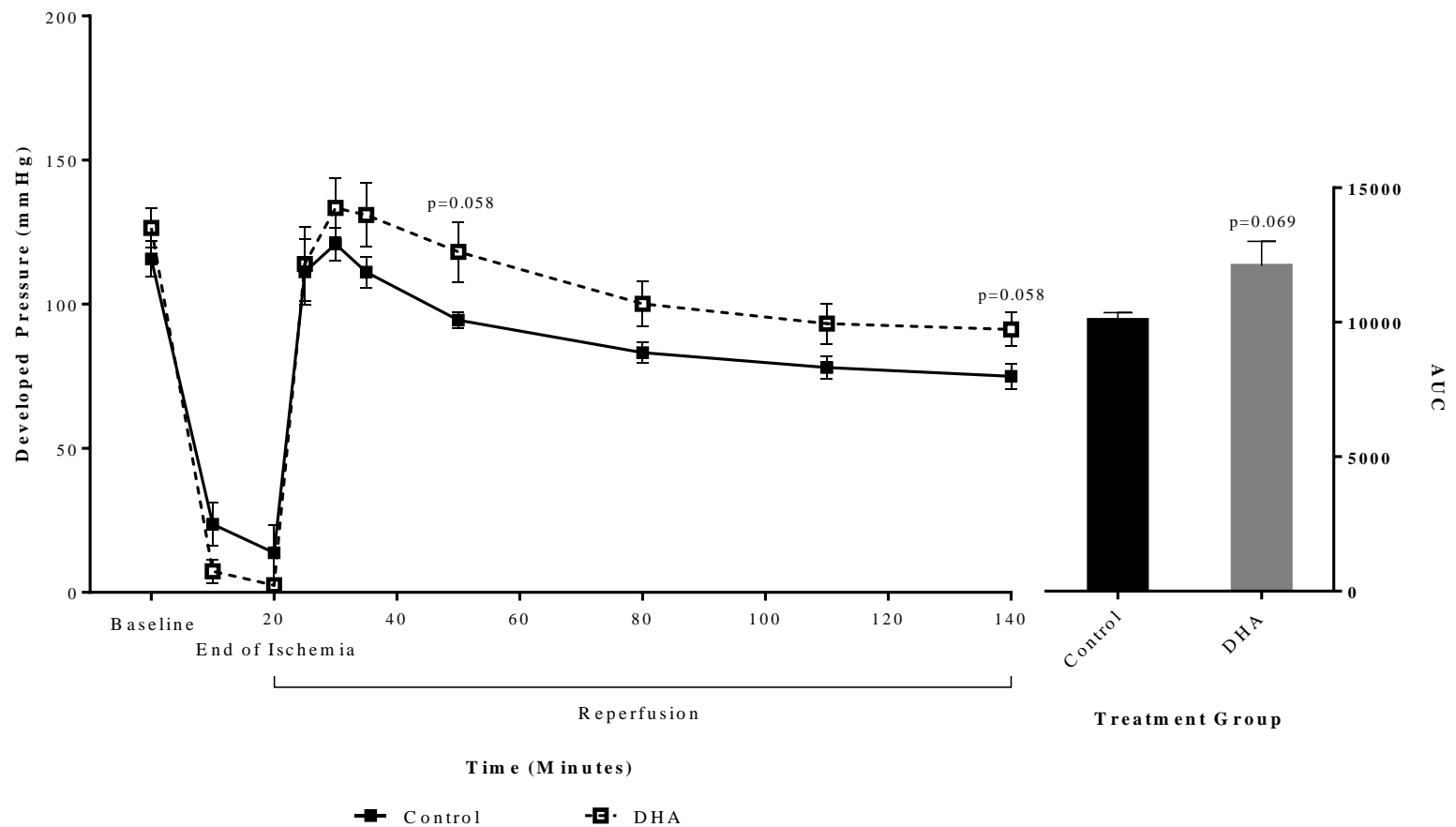


Figure 18. Left ventricular developed pressure in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Developed pressure is an index of the hearts ability to generate force and is calculated as the difference between systolic and diastolic pressure. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=5$).

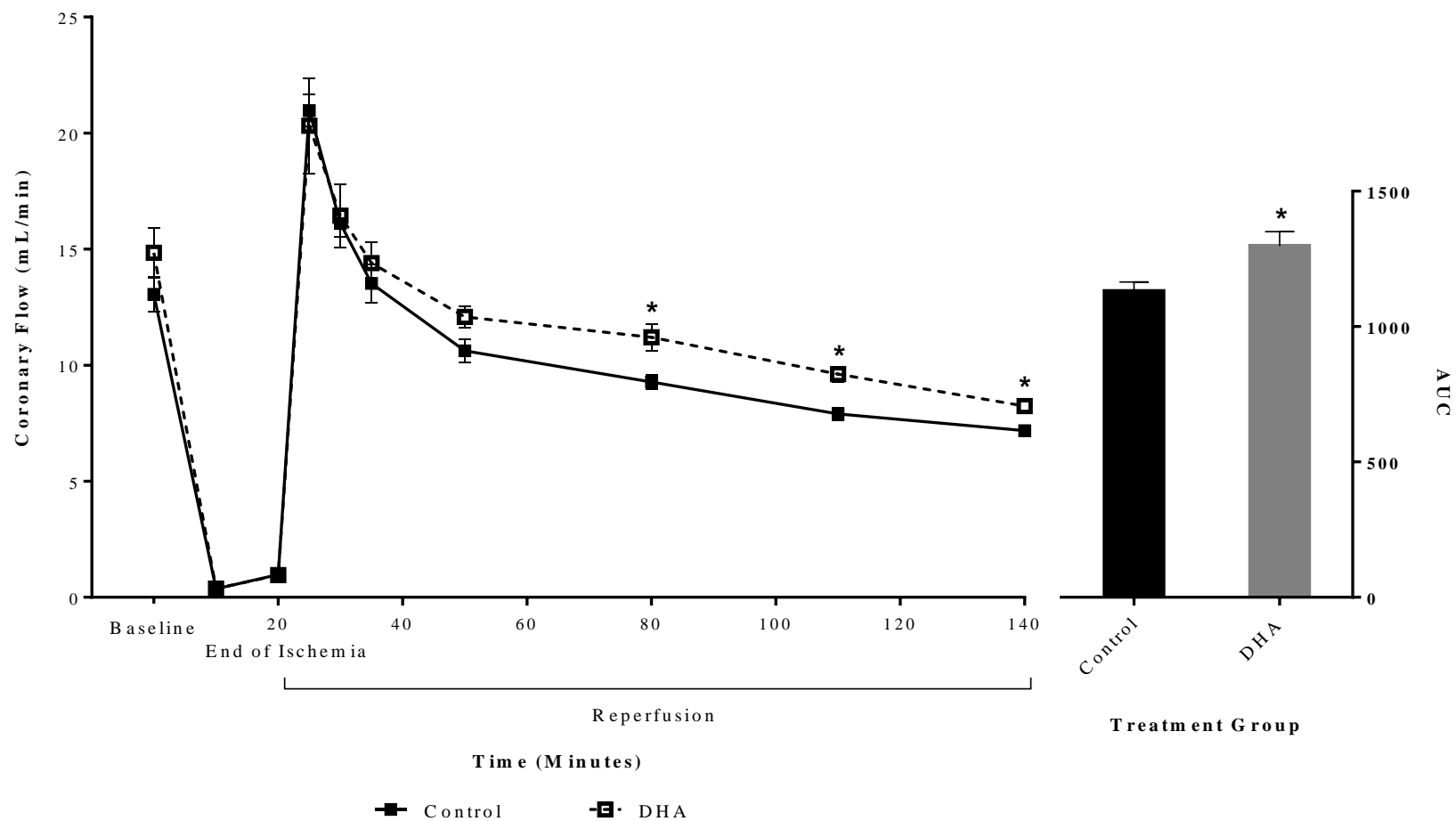


Figure 19. Coronary flow in millilitres per minute in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=5$).

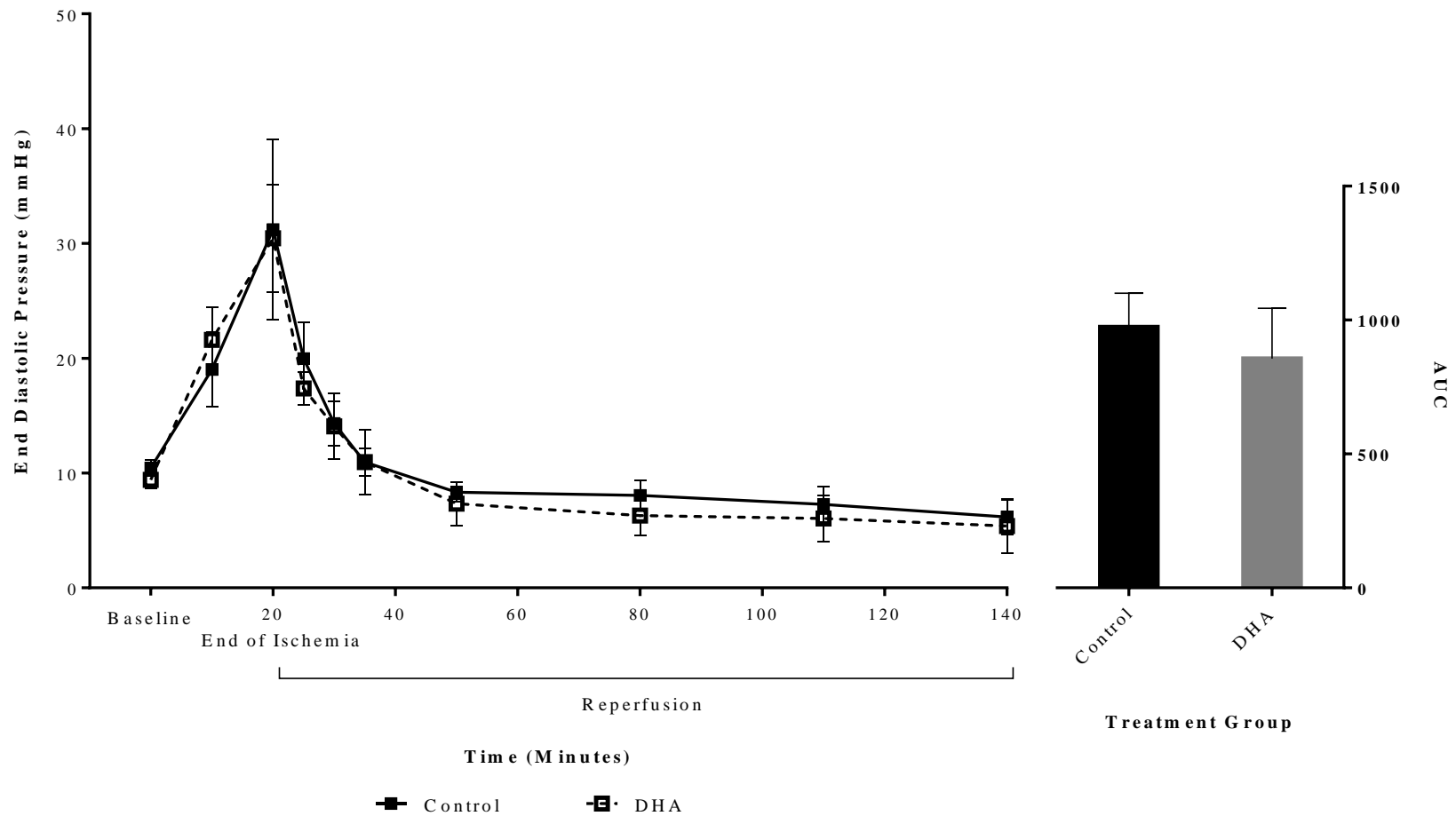


Figure 20. Left ventricular end diastolic pressure in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. Diastolic pressure is an index of cardiac damage, with higher values being associated with increased myocardial damage following ischemia. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. (n=5).

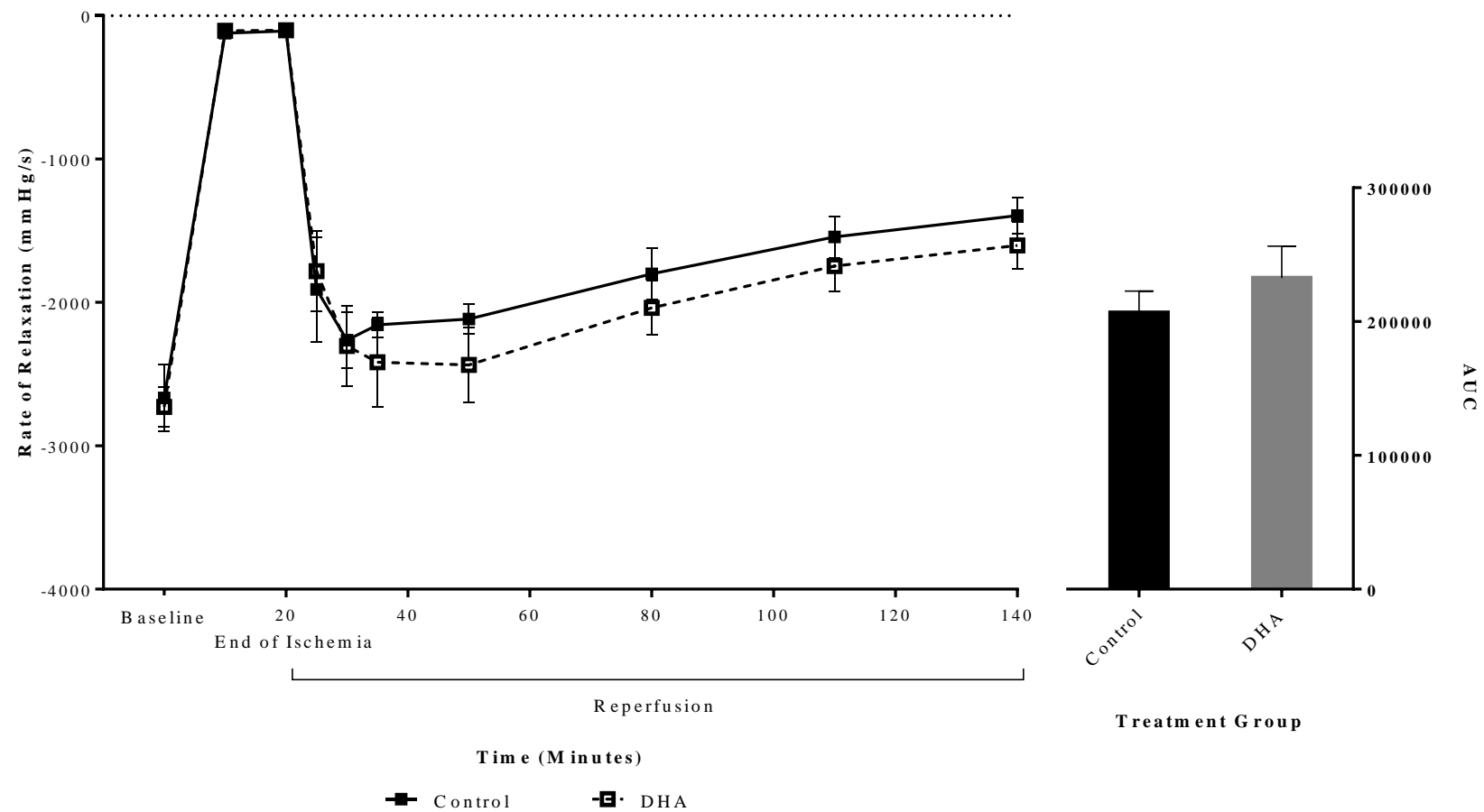


Figure 21. Left ventricular rate of relaxation in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=5$).

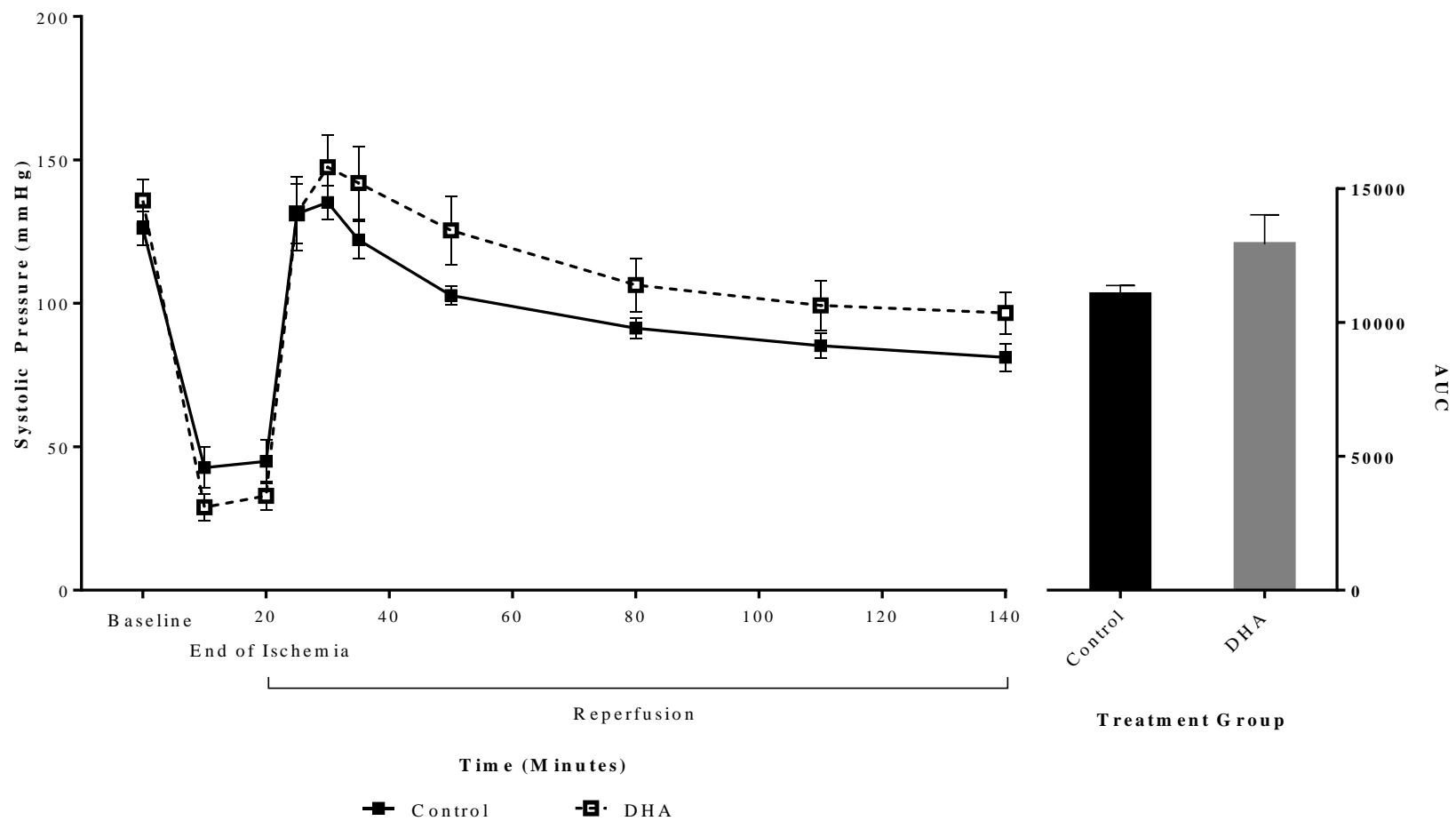


Figure 22. Left ventricular systolic pressure in male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. ($n=5$).

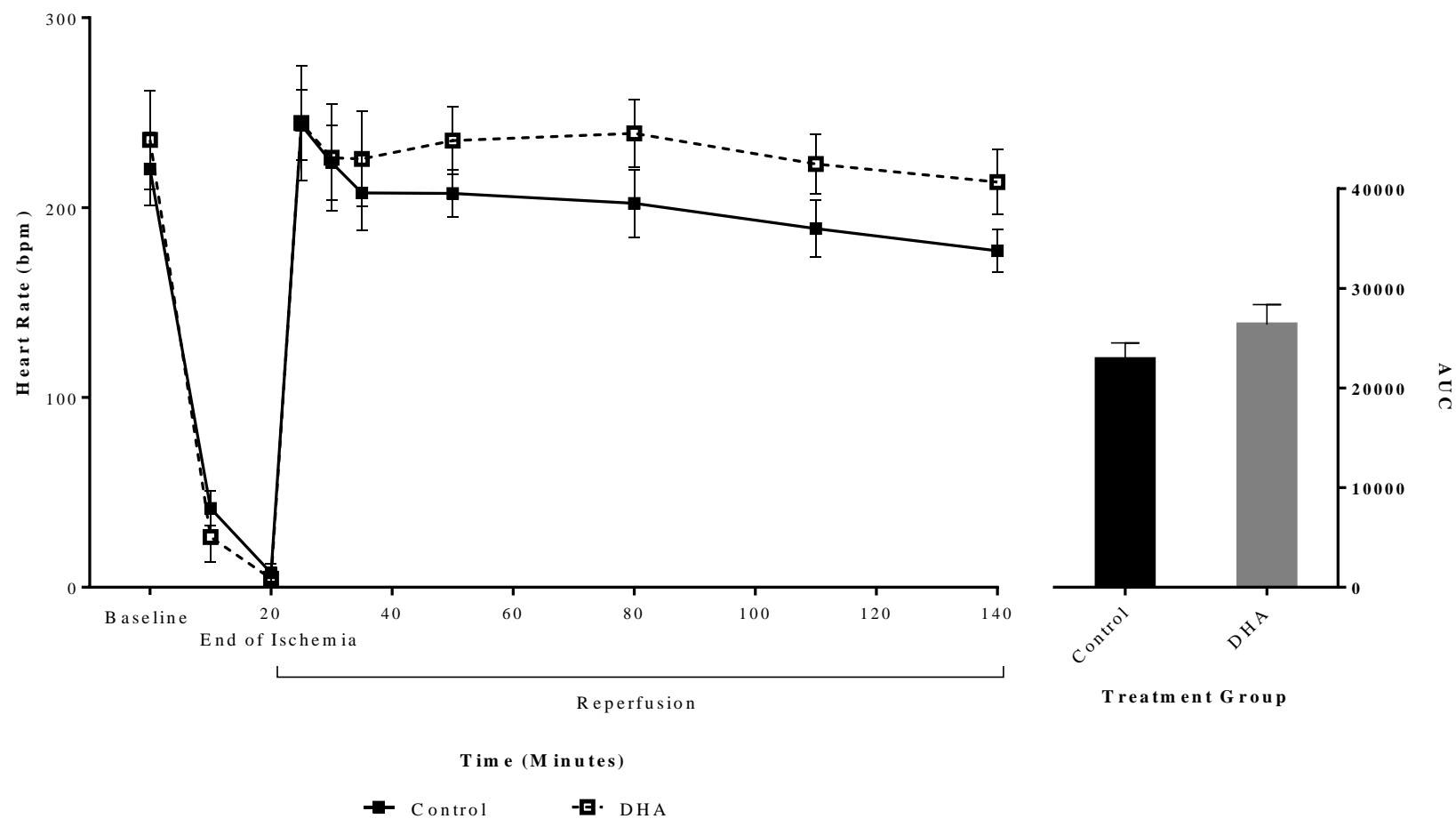


Figure 23. Heart rate in beats per minute of male Wistar rats exposed to either a DHA or control soybean oil gavage 24 hours before experimentation. * indicates significantly different ($p < 0.05$) from control at that timepoint by unpaired t-test. Values are expressed as mean \pm S.E.M. (n=5).

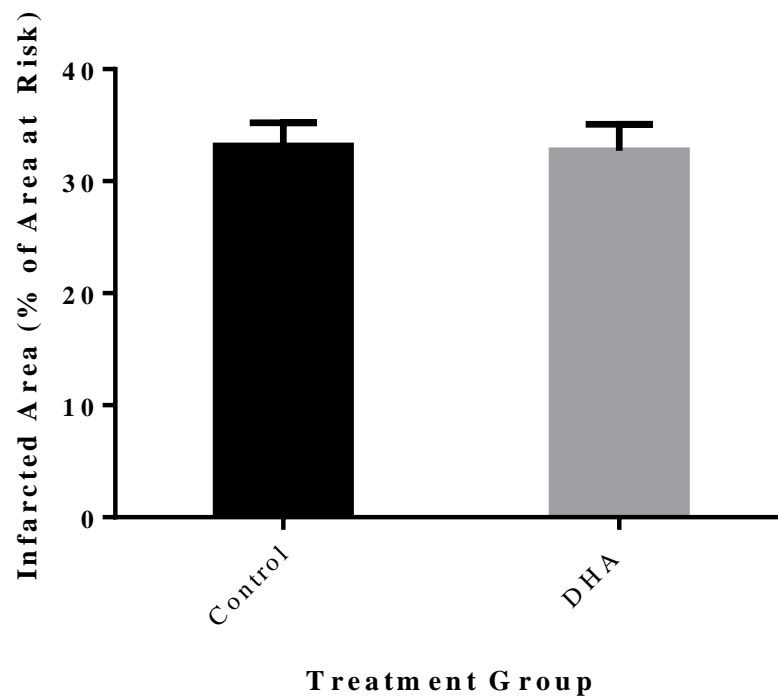


Figure 24. The effect of acute DHA intake on infarcted area in male Wistar rats. Data was analyzed by unpaired T-test. Values are expressed as mean \pm S.E.M.

Chapter 8

Effect of a Single DHA intake on the Myocardial Lipid Profile and Enzymatic Antioxidant Content

8.1. Introduction

In response to acute DHA feeding, male Wistar rats demonstrated increased plasma EPA and DHA along with increased left ventricular and coronary vascular function following ischemia. Previous feeding studies have implicated increased myocardial DHA (McGuinness et al. 2006) and enzymatic antioxidants (Castillo et al. 2013) in cardioprotection from ischemia-reperfusion injury. Therefore, these putative mechanisms that may explain the observed functional effect of acute DHA in male Wistar rats were investigated in a subset of animals.

8.2. Study Protocols

Standard chow fed male Wistar rats (3 – 5 months of age, n = 10) were gavaged with a single dose of DHA providing 0.4 mg DHA/g body weight or an equivalent volume of soybean oil as control. Following gavage, rats were returned to their cages and had ad libitum access to food and water for 12h and were then fasted for an additional 12h. Animals were then anaesthetized at 24 hours, and left ventricle tissue was collected.

A portion of left ventricular tissue was immediately homogenized in a glass homogenizer with cold phenylmethylsulfonyl fluoride (PMSF), and frozen at -80°C for enzymatic antioxidant analysis by western blotting. Total protein content was quantified by bicinchoninic acid assay. Samples containing 10 µg of protein were then separated in 10.5 - 12.5% polyacrylamide gels. Gels were then transferred to either nitrocellulose or polyvinylidene difluoride (BioRad Laboratories, Mississauga, Canada) by semi-dry transfer. Membranes were incubated overnight on a rocker at

4°C in 5% skim milk in 1x tris-buffered-saline with tween. Membranes were incubated with primary antibodies in milk for MnSOD (ADI-SOD-110), CuZnSOD (SOD-100), and GPx (sc-133160) according to supplier instructions for 1 hour. After application of horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), the enhanced chemiluminescence reagent ECL prime (GE Healthcare) was added to membranes. Membranes were visualized with the ChemiGenius2 Bioimaging System (Syngene inc., Frederick MD) and densitometry analysis was performed using Genesnap software (version 7.07). Molecular weights of proteins were identified using Precision Plus Protein WesternC Standards along with Precision Protein Strep-Tactin Horseradish Peroxidase Conjugated secondary antibody (Bio-Rad Laboratories). Equal protein loading was confirmed following analysis of target proteins with ponceau S stain (Bioshop, Burlington ON, representative Ponceau stains are found in Appendix A).

For fatty acid analysis, a portion of left ventricle was frozen at -80°C and pulverized in a mortar and pestle under liquid N₂. Pulverized samples were weighed and homogenized in chilled 2:1 chloroform methanol (v/v) containing 50 µg/ml butylated hydroxyl toluene (BHT) and docosatrienoic acid (C 22:3n-3) ethyl ester as an internal standard for absolute concentration determinations. Subsequently, 500 µL of 0.2 M Na₂PO₄ buffer was added and samples were inverted and centrifuged at 1734 *rcf* for 5 minutes. The organic phase was then collected and total lipid extracts were stored at -80°C until further analyses.

Total lipid extracts were analysed by direct methylation for total lipid composition as described in section 4.4. Additionally, left ventricular neutral lipids were separated into cholesteryl esters, non-esterified fatty acids, triacylglycerols, and total phospholipids by thin layer chromatography. Total lipid extracts were applied to 20 x 20 cm plates with a 6nm silica

gel layer (Whatman International LTD, Maidstone, England) and separated with a mobile phase of heptane:diethyl ether:glacial acetic acid (60:40:2 v/v/v) (Christie 2003). Bands were visualized under ultraviolet light using 2,7-dichlorofluorescein (Sigma-Aldrich, Oakville, ON, Canada) and were identified by comparison with reference standards. Bands were then scraped, collected into tubes, and lipids were extracted from silica gel shavings with 2:1 chloroform:methanol (Folch et al. 1957) and methylated as described above.

For lipidomic analysis to identify specific acyl species of lipids, pulverized tissue was independently weighed and homogenized in chilled 2:1 chloroform:methanol (v/v). All samples were spiked with 3.33 nmol of 17:0/17:0 glycerophosphatidylcholine and 3.33 nmol 17:0/17:0 glycerophosphatidylethanolamine dissolved in chloroform. Samples were left in solvents overnight to increase lipid recovery. A modified Folch double extraction was then performed as follows: after the addition of 500 μ L 0.2 M Na_2PO_4 in water, samples were inverted and centrifuged at 1734 *rcf* for 5 minutes. The organic phase was collected and an additional 2 mL of chloroform were added to the aqueous layer left in the original test tube. Samples were vortexed for 1 minute and re-centrifuged at 1734 *rcf* for 5 minutes. The resulting organic layer was extracted and combined with the first. Lipids were reconstituted in 100 μ L 65:35:5 acetonitrile:isopropanol:water (v/v/v) containing 0.1% formic acid. Samples were run on a Dionex UPLC system coupled to a Thermo Scientific Q-Exactive Orbitrap-Quadrupole Hybrid Mass Spectrometer. A binary, multi-step gradient was used, consisting of (A) 60:40 acetonitrile:water (v/v) and (B) 90:10 isopropanol:acetonitrile (v/v), both containing 10 mM ammonium formate and 0.1% formic acid. The flow rate was set at 260 μ L/min over a 47 minute gradient protocol with a C18 Ascentis Express column (15 cm x 2.1 mm x 2.7 μ m). The mass spectrometer was operated in positive electrospray ionization mode, with spray voltage of

3.0 kV, scan range of 200-2000 m/z , and under data-dependent acquisition conditions for the top 5 ions in MS/MS. MS/MS data was analyzed using the NIST 2.0 program, with the LipidBlast Database and the LipidMaps.org precursor ion search tool. A targeted precursor ion list was created prior to running the samples for the $[M+H]^+$ m/z values of all PC and PE glycerophospholipids containing DHA or EPA and even-chained fatty acids ranging from C 12:0 to C 24:1. Out of the 88 possible lipids that meet these criteria, 9 were confirmed in the samples (6 PC, 3 PE). There may be other glycerophospholipids containing EPA or DHA, however, due to factors such as ion suppression they were either not detectable or not verifiable by MS/MS. Ion profiles were extracted for the parent ions of the 9 lipids confirmed and the two lipid standards. Peaks were integrated and the area under the curve (AUC) for each peak was determined. All values were normalized relative to the AUC of the equivalent lipid standard.

8.3. Results

There was no significant effect of DHA treatment on animal body or heart weight ($p>0.05$). Left ventricular total fatty acids were affected by a single high DHA gavage (Table 6), but to a lesser extent than the plasma pool. The main myocardial lipid altered by DHA treatment was EPA, with concentrations increasing by 30% ($p<0.05$). There was a trend towards increasing DHA in the left ventricle with DHA gavage, but this did not reach statistical significance ($p=0.053$) due to high inter-individual variability in this pool. There were corresponding decreases in two saturated fatty acids, myristic (C 14:0) and lignoceric (C 24:0) acid.

When individual lipid classes of the left ventricle were isolated, several fatty acids were found to be higher in the DHA group as compared with control. Although DHA concentrations were elevated in both phospholipid (Table 7) and triacylglycerol (Table 8) pools, the former was

more significantly affected which is indicative of a preferential rapid incorporation of DHA into phospholipids over triacylglycerols. In phospholipids, both EPA ($p=0.024$) and DHA ($p=0.039$), were significantly elevated relative to control treated animals. Again, the increase in plasma eicosapentaenoic acid suggests DHA retro-conversion to EPA. In the triacylglycerol pool (Table 8), DHA ($p=0.011$), but not EPA ($p>0.05$), was elevated in DHA treated animals relative to control 24 hours following DHA feeding. The increase in left ventricular phospholipid and triacylglycerol DHA was not associated with a corresponding decrease in n-6 PUFAs ($p>0.05$). The DHA oil also contains high levels of saturated fatty acids (composition detailed in Table 1) which appeared to affect the left ventricular fatty acid composition by increasing 22:0 in the phospholipid pool and both 12:0 and 14:0 in the triacylglycerol pool ($p<0.05$).

There were no significant differences ($p>0.05$) in the concentration of either EPA or DHA in the phospholipid classes measured by lipidomic analysis (Table 9). There were, however, significant trends towards increasing total DHA ($p=0.081$) and DHA in phosphatidylethanolamine ($p=0.078$) fractions, primarily driven by increases in 16:0, 22:6n-3 PE. DHA concentrations were much greater in the phosphatidylethanolamine fraction than phosphatidylcholine. EPA concentrations, while observable in the phosphatidylcholine fraction, were minimal in phosphatidylethanolamine and therefore could not be quantified. Although increases in EPA and DHA were observed in total left ventricular phospholipids, lipidomic analysis did not implicate the myocardial phosphatidylcholine or phosphatidylethanolamine pools in this effect.

The myocardial protein content of the enzymatic antioxidants assayed, namely MnSOD, CuZnSOD, and GPx, was not significantly elevated 24 hours following a single DHA intake in male Wistar rats ($p>0.05$, Figure 25).

8.4. Discussion

A single DHA feeding resulted in increased myocardial DHA accretion in both the phospholipid and triacylglycerol pools, but not when measured in total lipid extract. Incorporation of EPA and DHA into the myocardial membrane has been implicated in cardioprotection (McGuinness et al. 2006) and may act through one or a combination of several pathways including alterations of membrane structure and function, lipid mediators of inflammation, and oxidative stress and upregulation of enzymatic antioxidants (discussed in Chapter 7).

Enzymatic antioxidants protect the heart from the damaging effect of ROS, including impairment of left ventricular function and induction of apoptotic cell death (Jones et al. 2003). This data did not support the hypothesis that DHA enhanced cardioprotection through the upregulation of enzymatic antioxidants, as the protein content of CuZnSOD, MnSOD, and GPx were not increased 24 hours after a single DHA feeding. It may be that longer term DHA treatment may be required to increase enzymatic antioxidant content. It has been demonstrated in chronic feeding studies ranging from 4 (Jahangiri et al. 2006) to 8 weeks in length (Castillo et al. 2013). It is also possible that, due to the time lag between digestion and incorporation into the myocardial membrane, 24 hours may not have provided enough time for the detection of increased enzymatic antioxidants. Following a preconditioning stimulus, the enzymatic antioxidant MnSOD was shown to rise slowly and did not peak until after 24 hours (Hoshida et al. 1993). Although no data is available on the time between a single DHA intake and myocardial incorporation, human studies have shown that n-3 HUFA does not peak in plasma until between 4 (Hanwell et al. 2009) and 6 (Harris et al. 2013) hours after ingestion. Therefore, any stimulus from DHA-induced oxidative stress may have not affected myocardial enzymatic

antioxidants until at least 4 to 6 hours after ingestion and an increase may not have been detectable until 24 hours after this stimulus. Additionally, the specific antioxidants measured may have affected whether or not an effect was observed. MnSOD has been implicated as a key contributor to the late phase of preconditioning and, as mentioned, it does not peak until 24 hours following the preconditioning stimulus (Hoshida et al. 1993). GPx, CAT, and MnSOD have been shown to increase following chronic dietary intervention (Jahangiri et al. 2006), although the time for this adaptation to occur is not known. HO-1 has been implicated in cardioprotection in several studies, and is related to 4-HXE levels through Nrf2 signalling (Nakagawa et al. 2014; Ishikado et al. 2013), but was not assayed in this study. It is possible that at 24 hours another assessment of upregulation of enzymatic antioxidants such as the nuclear translocation of Nrf2 or the mRNA of enzymatic antioxidants assayed by RT-PCR may have detected the early phases of upregulation. It is equally possible that at a later timepoint increases in enzymatic antioxidants may have been detectable. The lack of an increase in these enzyme antioxidants could possibly explain the lack of an effect of DHA treatment on infarction in male Wistar rats.

In conclusion, acute DHA feeding increases myocardial DHA accretion, particularly in the phospholipid and triacylglycerol pools. DHA does not increase the protein content of the enzymatic antioxidants CuZnSOD, MnSOD, and GPx and they are therefore not implicated in the effects on left ventricular function observed in this model. However, lacking a more comprehensive review of enzymatic antioxidant expression, it is impossible to state that their upregulation was not involved. Further assessment of other enzymatic antioxidants and different timepoints should be undertaken to determine if acute DHA feeding increases enzymatic antioxidants and was not detected in this particular model.

Table 6. Effect of Gavage on Left Ventricular Total Lipid Fatty Acid Concentration

Fatty Acid	$\mu\text{g fatty acid}/100 \text{ mg tissue}$	
	Control	DHA
C 10:0	nd	nd
C 12:0	0.2 ± 0.1	0.2 ± 0.0
C 14:0	5.1 ± 0.5	$4.1 \pm 0.4^*$
C 16:0	110.3 ± 3.6	107.0 ± 3.5
C 17:0	0.9 ± 0.1	0.9 ± 0.1
C 18:0	201.3 ± 11.6	196.2 ± 10.8
C 20:0	1.8 ± 0.3	1.7 ± 0.2
C 22:0	1.5 ± 0.2	1.5 ± 0.2
C 23:0	0.7 ± 0.0	0.6 ± 0.0
C 24:0	1.1 ± 0.4	$0.3 \pm 0.0^*$
Total SFA	329.6 ± 15.9	319.9 ± 13.4
C 12:1	nd	nd
C 14:1	nd	nd
C 16:1	1.2 ± 0.2	1.3 ± 0.2
C 18:1n-7	28.0 ± 1.3	26.7 ± 2.2
C 18:1n-9	29.9 ± 3.8	30.1 ± 7.0
C 20:1n-9	0.7 ± 0.1	0.7 ± 0.1
C 22:1n-9	0.5 ± 0.2	0.7 ± 0.1
C 24:1n-9	0.3 ± 0.1	0.3 ± 0.1
Total MUFA	64.4 ± 3.3	64.5 ± 8
C 18:2n-6	198.2 ± 31.0	189.3 ± 26.5
C 18:3n-6	1.2 ± 0.1	1.1 ± 0.1
C 20:2n-6	2.9 ± 0.2	2.5 ± 0.2
C 20:3n-6	3.3 ± 0.7	2.9 ± 0.3
C 20:4n-6	176.8 ± 13.9	172.2 ± 15.3
C 22:2n-6	0.2 ± 0.1	0.2 ± 0.1
C 22:4n-6	9.5 ± 1.5	8.9 ± 1.1
C 22:5n-6	6.2 ± 2.2	6.0 ± 1.3
Total n-6 PUFA	398.3 ± 41.3	383.2 ± 39.2
C 18:3n-3	2.1 ± 0.3	1.8 ± 0.2
C 20:3n-3	0.2 ± 0.1	0.2 ± 0.1
C 20:5n-3	1.0 ± 0.1	$1.3 \pm 0.1^*$
C 22:5n-3	27.8 ± 2.3	25.0 ± 1.9
C 22:6n-3	110.7 ± 8.7	$125.4 \pm 11.5^{(p=0.053)}$
Total N-3 PUFA	141.8 ± 8.0	153.7 ± 12.3
Total Fatty Acids	934.0 ± 54.3	921.4 ± 50.4

Data is expressed as mean \pm SD (n = 5 per group) * indicates significantly different from controls (p < 0.05) by independent t-test. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, nd: not detected.

Table 7. Effect of Gavage on Left Ventricular Phospholipid Fatty Acid Concentration

Fatty Acid	µg fatty acid/100 mg tissue	
	Control	DHA
C 10:0	0.2 ± 0.1	nd
C 12:0	1.4 ± 0.3	1.6 ± 0.2
C 14:0	5.4 ± 1.1	7.7 ± 0.4
C 16:0	129.8 ± 5.7	146.9 ± 8
C 18:0	271.7 ± 12.5	291.7 ± 17
C 20:0	2.9 ± 0.1	3.0 ± 0.1
C 22:0	2.1 ± 0.2	2.8 ± 0.1*
C 24:0	0.6 ± 0.1	0.8 ± 0.2
Total SFA	414.0 ± 17.6	454.6 ± 24.8
C 12:1	0.7 ± 0.2	1.3 ± 0.2
C 14:1	0.1 ± 0.1	0.6 ± 0.5
C 16:1	2.6 ± 0.1	4.0 ± 0.1*
C 18:1n-7	32.9 ± 1.1	34.0 ± 2.6
C 18:1n-9	32.4 ± 4.0	32.7 ± 2.3
C 20:1n-9	1.6 ± 0.2	1.4 ± 0.1
C 22:1n-9	0.9 ± 0.1	1.1 ± 0.1
C 24:1n-9	0.3 ± 0.1	0.3 ± 0.1
Total MUFA	71.4 ± 5.0	75.3 ± 5.1
C 18:2n-6	223.8 ± 20.5	243.1 ± 15.5
C 18:3n-6	0.3 ± 0.1	0.3 ± 0.1
C 20:2n-6	2.9 ± 0.1	2.8 ± 0.2
C 20:3n-6	3.0 ± 0.2	3.1 ± 0.2
C 20:4n-6	188.6 ± 10.9	200.6 ± 16.4
C 22:2n-6	0.2 ± 0.1	0.3 ± 0.1
C 22:4n-6	9.0 ± 0.8	9.1 ± 0.5
C 22:5n-6	5.7 ± 0.9	6.2 ± 0.8
Total n-6 PUFA	433.4 ± 31.2	465.4 ± 31.2
C 18:3n-3	1.5 ± 0.1	1.6 ± 0.1
C 20:3n-3	0.5 ± 0.1	0.5 ± 0.0
C 20:5n-3	0.9 ± 0.1	1.2 ± 0.1*
C 22:5n-3	25.1 ± 1.9	25.6 ± 2.7
C 22:6n-3	106.6 ± 3.4	130.2 ± 9.0*
Total N-3 PUFA	134.6 ± 4.5	159.0 ± 11.7
Total Fatty Acids	1053.4 ± 54.0	1154.4 ± 65.8

Data is expressed as mean ± SD (n = 5 per group) * indicates significantly different from controls (p < 0.05) by independent t-test. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, nd: not detected.

Table 8. Effect of Gavage on Left Ventricular Triacylglycerol Fatty Acid Concentration

Fatty Acid	$\mu\text{g fatty acid}/100 \text{ mg tissue}$	
	Control	DHA
C 10:0	0.1 ± 0.1	0.1 ± 0.1
C 12:0	0.4 ± 0.1	$1.0 \pm 0.2^*$
C 14:0	1.9 ± 0.2	$3.0 \pm 0.4^*$
C 16:0	34.1 ± 1.9	39.4 ± 3.8
C 18:0	21.5 ± 0.5	24.7 ± 2.2
C 20:0	0.9 ± 0.1	1.1 ± 0.1
C 22:0	0.7 ± 0.1	0.9 ± 0.1
C 24:0	0.1 ± 0.0	0.4 ± 0.1
Total SFA	59.7 ± 2.4	70.6 ± 5.7
C 12:1	0.1 ± 0.1	0.1 ± 0.1
C 14:1	0.1 ± 0.1	0.1 ± 0.1
C 16:1	3.6 ± 0.3	4.3 ± 0.2
C 18:1n-7	3.9 ± 0.3	4.5 ± 0.5
C 18:1n-9	16.2 ± 1.8	19.2 ± 3.3
C 20:1n-9	0.4 ± 0.1	0.3 ± 0.0
C 22:1n-9	0.7 ± 0.1	1.0 ± 0.1
C 24:1n-9	0.1 ± 0.1	0.1 ± 0.1
Total MUFA	25.1 ± 2.4	29.5 ± 3.7
C 18:2n-6	24.3 ± 2.8	24.9 ± 5.5
C 18:3n-6	0.3 ± 0.1	0.2 ± 0.1
C 20:2n-6	0.5 ± 0.1	0.4 ± 0.1
C 20:3n-6	0.5 ± 0.1	0.4 ± 0.1
C 20:4n-6	3.9 ± 0.2	4.0 ± 0.3
C 22:2n-6	0.3 ± 0.1	0.3 ± 0.1
C 22:4n-6	0.9 ± 0.1	0.8 ± 0.1
C 22:5n-6	0.2 ± 0.1	1.9 ± 1.5
Total n-6 PUFA	30.9 ± 3.3	32.9 ± 6.1
C 18:3n-3	0.7 ± 0.1	0.7 ± 0.2
C 20:3n-3	0.5 ± 0.1	0.6 ± 0.0
C 20:5n-3	0.3 ± 0.1	0.4 ± 0.1
C 22:5n-3	1.5 ± 0.2	1.4 ± 0.1
C 22:6n-3	2.4 ± 0.3	$3.9 \pm 0.3^*$
Total N-3 PUFA	5.3 ± 0.6	7.0 ± 0.5
Total Fatty Acids	121.0 ± 8.3	140.0 ± 14.6

Data is expressed as mean \pm SD (n = 5 per group) * indicates significantly different from controls (p < 0.05) by independent t-test. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, nd: not detected.

Table 9. Effect of Gavage on n-3 HUFA in Left Ventricular Phospholipid Acyl-Species

Phospholipid Class	Sn-1 Acyl	Sn-2 Acyl	Concentration (nmol/100 mg)	
			Control	DHA
Phosphatidylcholine	16:0	20:5n-3	3.4 ± 0.5	3.0 ± 0.1
	18:0	20:5n-3	4.0 ± 0.7	3.8 ± 0.3
	Sum 20:5n-3 PC		7.4 ± 1.2	6.8 ± 0.4
	16:0	22:6n-3	39.6 ± 3.3	61.1 ± 10.1
	18:0	22:6n-3	61.9 ± 5.0	72.4 ± 10.8
	18:1	22:6n-3	1.8 ± 0.1	3.0 ± 0.6
Phosphatidylethanolamine	18:2	22:6n-3	7.3 ± 1.0	10.9 ± 1.7
	Sum 22:6n-3 PC		110.6 ± 7.4	147.4 ± 23.1
	16:0	22:6n-3	230.1 ± 20.7	315.8 ± 37.5
	18:1	22:6n-3	45.0 ± 3.8	58.9 ± 5.9
	18:2	22:6n-3	34.5 ± 3.1	49.0 ± 7.4
	Sum 22:6n-3 PE		309.6 ± 26.2	423.7 ± 50.1

Data is expressed as mean ± SEM (n = 5 per group). Data was analysed by independent t-test. PC: phosphatidylcholine; PE: phosphatidylethanolamine.

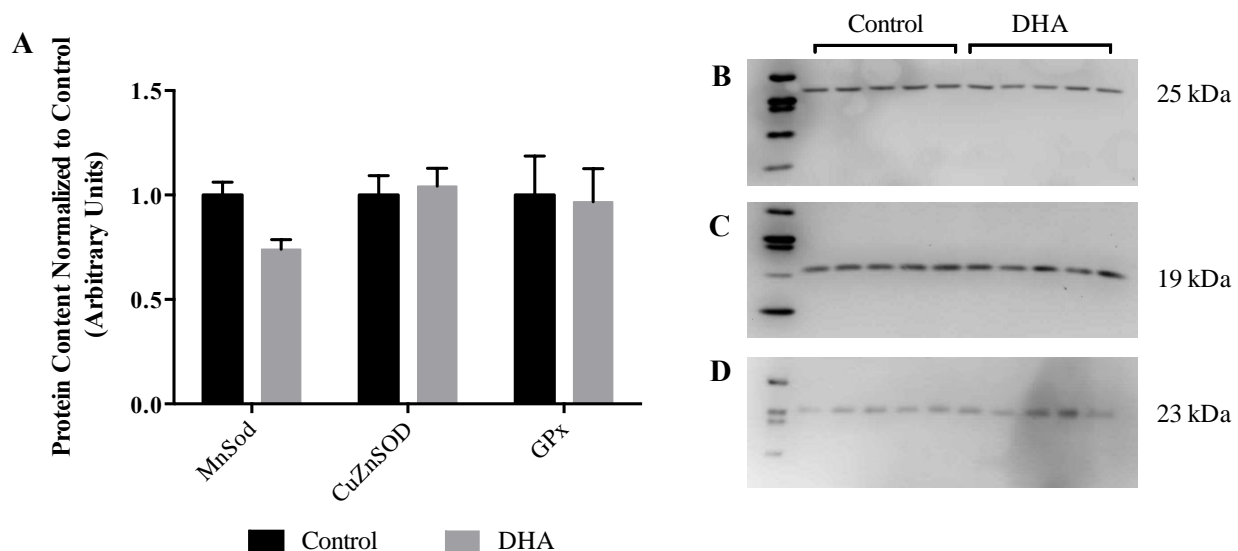


Figure 25. The effect of DHA treatment on the protein content of enzymatic antioxidants in the left ventricle (A). Values are expressed as mean ± S.E.M. (n=5). Representative blots of MnSOD, CuZnSOD, and GPx are shown in figures B, C, and D, respectively.

Chapter 9

Discussion

DHA has been implicated in cardioprotection in both epidemiological studies (Albert et al. 2002) and laboratory investigations in animals (Zeghichi-Hamri et al. 2010), and one of the cardioprotective mechanisms appears to involve the reduction of ischemia-reperfusion injury (Castillo et al. 2013; Zeghichi-Hamri et al. 2010). Ischemia-reperfusion injury results in myocardial functional impairment and infarction, which is caused in large part by ROS (Kim et al. 2006; Temsah et al. 1999) but can be alleviated with antioxidant enzymes (Das et al. 1992; Jones et al. 2003). Chronic dietary intervention with EPA and DHA increases cellular antioxidant enzymes (Jahangiri et al. 2006) and affords protection from ischemia-reperfusion injury (Castillo et al. 2013), possibly through detoxification of ROS. Counterintuitively, EPA and DHA feeding increases oxidative stress (Vossen et al. 1995), and DHA is susceptible to lipid peroxidation (Saito & Kubo 2003). However, products of peroxidation increase the expression and protein content of enzymatic antioxidants, and this adaptation can occur in less than 24 hours (Zhang et al. 2010). The ability of DHA to effect changes in myocardial susceptibility to ischemia reperfusion injury in this time frame has not been characterized. Therefore this thesis evaluated the effect of acute DHA intake on susceptibility to ischemia-reperfusion injury, myocardial DHA accretion, and enzymatic antioxidant content.

A single dose of DHA affected left ventricular function at 24 hours in both strains and perfusion methods studied, although the effect of DHA differed between the two models. DHA feeding lowered baseline left ventricular function in male Sprague Dawley rats perfused at constant flow, but did not cause comparable baseline impairment in male Wistar rats perfused at

constant pressure. Sprague-Dawley rats demonstrated reduced left ventricular function in the reperfusion period, but increased recovery relative to baseline levels. Wistar rats exhibited some degree of improved left ventricular function following ischemia-reperfusion injury, but not the same extent as Sprague-Dawley male rats. There were no differences in the coronary perfusion pressure in male Sprague-Dawley rats, while male Wistar rats had higher coronary flow following ischemia with acute DHA feeding. The literature surrounding the effect of EPA and DHA treatment on left ventricular functional recovery following ischemia-reperfusion injury is mixed, with some studies having found improved recovery from myocardial ischemia-reperfusion (Castillo et al. 2013; Abdukeyum et al. 2008) and others detecting no difference in recovery (Force et al. 1989; Zeghichi-Hamri et al. 2010; McGuinness et al. 2006). Myocardial infarction, as measured by TTC staining, also was strain-dependent. A single DHA feeding reduced infarction in Sprague-Dawley rats independent of sex. Conversely, there was no effect of DHA on infarction in male Wistar rats. Evidence in the literature is again mixed as to the role of DHA in reducing myocardial infarction, with some studies describing a reduction in myocardial infarction (Castillo et al. 2013; Zeghichi-Hamri et al. 2010) while others have reported no effect (Force et al. 1989).

The coronary flow response may also explain the different results observed in the two DHA treatment models. In constant pressure perfusion, there was an approximately 30% increase in flow relative to baseline observed immediately following ischemia independent of treatment group, a phenomenon termed reactive hyperemia (Khanamiri et al. 2013). In providing increased flow, the small effect previously observed with DHA treatment on infarction in constant flow studies may have been masked, as reactive hyperemia may actually increase the degree of myocardial damage during reperfusion (Olivecrona et al. 2007). In constant flow perfusion,

reactive hyperemia does not occur, and could potentially explain the observation of an effect of DHA on myocardial infarction in this model.

The female response to DHA treatment differed markedly from males. Female hearts of Sprague-Dawley rats did not demonstrate increased recovery of any left ventricular functional parameters with DHA treatment. Baseline left ventricular functional differences with treatment, which were apparent in males, also did not occur in female hearts. Therefore acute DHA intake did not affect the left ventricular function of female hearts, and did not result in increased protection from ischemia-reperfusion injury. One possible explanation is that DHA-mediated alterations in left ventricular function may not have any effect in females due to their innately higher myocardial DHA content (Kitson et al. 2012). Females also have increased phosphorylation and activity of the antioxidant enzyme aldehyde dehydrogenase (ALDH2) (Lagranha et al. 2010) along with increased myocardial n-3 HUFA content, which has been shown to increase cellular oxidative stress defence through the upregulation of enzymatic antioxidants (Jahangiri et al. 2006; Castillo et al. 2013).

Acute DHA intake resulted in altered myocardial and plasma lipid profiles after 24 hours. Previous human studies have demonstrated plasma level increases 24 hours following a single n-3 HUFA intake (Yang et al. 2012), but in the animal ischemia-reperfusion injury literature chronic dietary interventions are typically used and this is the first study to demonstrate myocardial EPA and DHA accretion 24 hours following a single DHA feeding. It was hypothesized that in this acute time frame DHA would increase principally in plasma, and thus any effects on the cardiovascular system would be mediated by free DHA and not membrane DHA. Only total fatty acids were analyzed in the plasma pool and therefore conclusions cannot be drawn as to the molecular form of DHA within plasma. However, inferences can be made

from human studies, which have demonstrated DHA increases peak in the plasma phospholipid pool at 24 hours (Raatz et al. 2009) while red blood cell incorporation is comparatively delayed (Metherel et al. 2009).

The effect of acute DHA intake on myocardial lipids was not immediately evident, as the DHA concentration was not significantly elevated in myocardium total lipids after 24 hours (Table 6). However, DHA was found to be elevated in both the phospholipid (Table 7) and triacylglycerol (Table 8) pools. DHA is preferentially incorporated into phospholipids, and particularly PE (Kubo et al. 2000). An increase in triacylglycerol DHA is interesting as this is not typically thought to be a site of DHA accumulation. Previous studies have demonstrated myocardial DHA accretion following more prolonged n-3 HUFA intake of between 4 (Jahangiri et al. 2006) and 8 weeks (Castillo et al. 2013).

As the DHA source used contained trace amounts of EPA (Table 3) it was hypothesized that there would be minimal EPA accumulation in plasma and left ventricle. However, EPA concentrations in plasma and myocardial lipids increased significantly, suggesting that the DHA provided was retroconverted to EPA. This is the first observation of increased absolute EPA concentrations in the heart lipid pools after a single oral dose of DHA. DHA conversion to EPA has been observed in longer term human supplementation studies (Stark & Holub 2004) and radioisotope tracer studies examining plasma have indicated that DHA retroconversion to EPA can occur after a single dose of DHA in both rats and humans (Brossard et al. 1996). It is believed that this occurs through β -oxidation in the peroxisome (Stark & Holub 2004). Therefore the response to DHA feeding may include effects of EPA on cardiovascular function. EPA is a precursor to eicosanoids and competes directly with ARA for phospholipid incorporation. EPA is a precursor to anti-inflammatory lipid mediators (Ohnishi & Saito 2013),

and has demonstrated anti-inflammatory properties in cardiovascular disease (Cawood et al. 2010). Because of retroconversion, separating the specific effects of EPA and DHA through dietary treatment may be very difficult and it therefore may be necessary to directly infuse DHA into the heart to examine the individual effects of EPA and DHA.

The protein content of the enzymatic antioxidants MnSOD, CuZnSOD, and GPx was not significantly altered 24 hours following DHA treatment, indicating that these particular antioxidant enzymes are not implicated in the observed effects on left ventricular and coronary vascular function observed in this model. However, lacking a more comprehensive review of enzymatic antioxidant expression including assessment in Sprague-Dawley rats, it is impossible to state that their upregulation was not involved. Unfortunately, these enzymes were not measured in the constant flow model where an infarction effect was observed. Other studies, employing longer term EPA and DHA supplementation protocols, have demonstrated increased expression and/or protein content of enzymatic antioxidants in murine models (Jahangiri et al. 2006; Castillo et al. 2013).

Although it was hypothesized that DHA increased oxidative stress following acute intake and would result in increased expression of enzymatic antioxidants, oxidative stress and all potential enzymatic antioxidants were not assayed in these studies. However, previous studies have demonstrated that chronic dietary intervention with n-3 HUFA results in increased oxidative stress in several models studied including endothelial cells (Vossen et al. 1995), plasma (Song et al. 2000), liver (Farina et al. 2003), kidney (Ibrahim et al. 1999), and human blood biomarkers (Garcia-Alonso et al. 2012). Increased oxidative stress is detrimental to cardiovascular function, and this effect is partially mediated by reactive aldehydes, which are a product of fatty acid peroxidation. 4-hydroxy-2-nonenal (4-HNE) is a product of the non-enzymatic oxidation of

ARA, and it has been shown to cause cell death at concentrations $>10\mu\text{M}$ in isolated cardiomyocytes (Zhang et al. 2010). However, these products may also be cardioprotective, as they are ligands for transcription factors including Nrf2 which controls downstream expression of several genes related to oxidative stress and the inflammatory response (Zhang et al. 2010; Ishikado et al. 2013). The n-3 derived lipid peroxidation product, 4-hydroxyhexenal has been shown to increase the enzymatic antioxidants catalase, GSTA1, and GCLC (Anderson et al. 2012) and HO-1 (Nakagawa et al. 2014) in the murine heart.

As there were no appreciable adaptations in the antioxidant system detected in these studies, myocardial EPA and DHA accretion may be acting through another mechanism in order to cause changes in left ventricular and coronary vascular function. This may include alterations in membrane structure and function, as incorporation of n-3 HUFA into myocardial phospholipids affects the function of numerous signal transduction pathways originating at the membrane. Inhibition of toll-like receptor 4 (TLR4), a receptor mediator of the inflammatory response in the plasma membrane, by EPA and DHA has significant downstream effects including the inhibition of lipopolysaccharide-induced inflammation, COX-2 expression, and NF κ B activation (Lee et al. 2003). This has been attributed to the ability of DHA to inhibit the lipid-raft dependent dimerization and recruitment of TLR4, which is a critical step in the signalling pathway (Wong et al. 2009). Another inflammatory signalling pathway, PKC θ , was found to be inhibited in animals fed diets enriched in either fish oil or DHA alone resulting in inhibition of downstream inflammatory responses including NF κ B activation and interleukin-2 secretion (Fan et al. 2004). It is thought that DHA incorporation into plasma membrane phosphatidylcholine and phosphatidylethanolamine and a corresponding reduction in lipid raft sphingomyelin was responsible for disruption of this signalling pathway.

Observed effects may also be attributable to DHA-derived lipid mediators, as DHA in myocardial phospholipids serves as a precursor to resolvins and protectins which may have roles in cardioprotection. Resolvin D1 has been shown to activate inflammation resolution following myocardial infarction, resulting in improved ventricular function (Kain et al. 2015). DHA displaces ARA in the myocardial membrane, and this may reduce some of the proinflammatory effects of ARA and its derivatives, which has been shown to promote inflammation and cause left ventricular dysfunction following a myocardial infarction (Kain et al. 2014).

Myocardial DHA may also affect hemodynamic function through ion channel modulation. During ischemia, the activity of plasmalogen-specific phospholipase A₂ is increased (Ford et al. 1991), resulting in cleavage of fatty acyls including n-3 HUFA from the sn-2 position of plasma membrane phospholipids. This pool of liberated n-3 HUFA could potentially exert anti-arrhythmic effects through direct effects on myocardial ion channels including the voltage gated L-type Ca²⁺ channel (Xiao et al. 1997) and the alpha subunit of the sodium channel (Xiao et al. 2001). Isolated heart studies have demonstrated myocardial protection from ischemia-reperfusion injury with infusion of n-3 HUFA directly into the perfusate likely demonstrating an effect of DHA in the free fatty acid pool (Smith et al. 2012; Richard et al. 2014).

9.1. Limitations and Future Directions

Issues with the models used in these studies may limit the interpretation of findings and their applicability to human clinical populations. However, further study can be undertaken in order to increase understanding of the mechanistic role of acute DHA intake in the findings of these studies. It will be important to fully characterize the biochemical and molecular pathways that pertain to the observed reductions in left ventricular function, improved post-ischemic contractile

recovery, and elevated reperfusion coronary flow, as well as the evident strain and perfusion differences.

The Langendorff isolated heart model is an excellent model for mechanistic study of the heart, in that it facilitates the isolation of the myocardium from neural and hormonal confounders. By utilizing constant pressure perfusion, the physiological relevance is increased as the flow rate is regulated by the coronary vasculature as it is *in vivo*. However, the isolated heart is still a non-physiological model, and application to *in vivo* situations may be limited. The rat is a common model used for cardiovascular study, although differences between the cardiac function in humans and rats may limit application of some findings. Of note, the Ca^{2+} sequestration during diastole differs significantly between humans and rats, with rats relying more heavily on SERCA activity than humans (Bers 2002). Additionally, the dose of DHA used may not be clinically applicable. Although equivalent high doses of n-3 HUFA have been used in both murine models (Zeghichi-Hamri et al. 2010) and human studies (Endres et al. 1989), such a high dose may not be feasible in human populations due to both tolerance of adverse side-effects and cost.

Studying the effect of acute DHA treatment using both heart perfusion techniques and 2 animal strains provided insight into its effect on the cardiovascular system. However, as these factors were not studied independently but were rather changed simultaneously for reasons of physiological relevance and increased ischemic susceptibility, it is difficult to definitively discuss the independent effect of either rat strain or perfusion method on ischemia-reperfusion injury. Assays that were performed in Wistar rats, namely myocardial DHA accretion and enzymatic antioxidants, were not performed in Sprague-Dawley rats and again make comparison between the 2 strains difficult. Therefore, strain differences in the molecular targets of

preconditioning should be characterized more fully, as they may contribute to the differential response in Sprague-Dawley and Wistar rats. These include differences in enzymatic antioxidants and myocardial DHA accretion.

EPA and DHA are often studied in combination in animal and human studies, which complicates interpretation of experimental results as it is unclear whether the fatty acids act individually or in combination. DHA was used for investigations in this thesis due to its increased susceptibility to peroxidation relative to EPA, as it was hypothesized that the n-3 HUFA would act through oxidative stress pathways to reduce ischemia-reperfusion injury. It is unknown at this point what effect EPA or EPA + DHA treatment would have on ischemia-reperfusion injury 24 hours later. Although insights gained from this work indicate a potential effect of acute DHA feeding on cardiovascular function, application to human populations may be limited as EPA and DHA are often found together in whole foods and nutraceuticals. The n-3 HUFA EPA, although less susceptible to peroxidation than DHA, may still elicit a similar myocardial response following acute intake. In fact, these results suggest that increased EPA in the myocardium with DHA treatment may actually be contributing to cardioprotection from ischemia-reperfusion injury in this model. Therefore the effect of EPA individually and in combination with DHA should be tested.

Although enzymatic antioxidants were assayed in this study, other known physiological effects of DHA on the cardiovascular system including, but not limited to, ion channel kinetics, membrane structure and function, and lipid mediator effects were not measured. Therefore, their role in these observations should be investigated further. It is also not clear at this time if the dose used in these studies caused an increase in myocardial oxidative stress, and if this signal acted through traditional preconditioning pathways such as the ROS-sensitive transcription factor

Nrf2. This could explain the lack of an effect on enzymatic antioxidant content, and therefore the oxidative stress in the myocardium following DHA intake should be characterized, including the role of lipid peroxidation and the formation of the reactive aldehyde 4-HNE. The extent of Nrf2 pathway activation in response to oxidative stress, as well as the expression and content of its downstream antioxidant targets, including GSTA1 and GCLC, should also be characterized.

9.2. Conclusions

These results demonstrate that a single oral dose of DHA affects the function of the isolated rat heart just 24 hours following intake. At baseline, DHA resulted in reduced left ventricular contractility in male Sprague-Dawley rats, although this effect was dependent on both animal sex and strain, as female heart function and the baseline function of hearts from Wistar rats were not affected by DHA treatment. DHA intake also affected the myocardial response to ischemia-reperfusion injury, as male Sprague-Dawley and Wistar rats demonstrated improved recovery of left ventricular function during reperfusion, with DHA-treated Wistar rats also demonstrating increased reperfusion coronary flow. These functional changes are associated with an altered plasma total lipid and left ventricular phospholipid profile, with significant increases in both the n-3 HUFA EPA and DHA and, in the case of plasma total lipid, a corresponding decrease in the relative percent of the n-6 HUFA ARA. Although oxidative stress pathways may alter the enzymatic antioxidant content of the myocardium resulting in cardioprotection, there was no evidence for the involvement of MnSOD, CuZnSOD, or GPx in this model as protein levels of these enzymes were not increased 24 hours following DHA treatment. Taken together, these results suggest that acute DHA intake modulates the baseline left ventricular function, coronary vascular tone, and ischemic susceptibility of the myocardium 24 hours later, but these effects are

dependent on strain, sex, and perfusion method. These alterations were associated with myocardial EPA and DHA accretion, particularly in myocardial phospholipids, but not an increased content of the enzymatic antioxidants assayed.

References

- Abdukeyum, G.G., Owen, A.J. & McLennan, P.L., 2008. Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning. *J.Nutr.*, 138(1541-6100 (Electronic)), pp.1902–1909.
- Adlam, V.J. et al., 2005. Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *FASEB J.*, 19(1530-6860 (Electronic)), pp.1088–1095.
- Aires, V. et al., 2007. Docosahexaenoic acid induces increases in $[Ca^{2+}]_i$ via inositol 1,4,5-triphosphate production and activates protein kinase C gamma and -delta via phosphatidylserine binding site: implication in apoptosis in U937 cells. *Mol.Pharmacol.*, 72(1521-0111 (Electronic)), pp.1545–1556.
- Albert, C.M. et al., 2002. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *The New England journal of medicine*, 346(15), pp.1113–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11948270> [Accessed July 19, 2014].
- Anderson, E.J. et al., 2012. Aldehyde stress and up-regulation of Nrf2-mediated antioxidant systems accompany functional adaptations in cardiac mitochondria from mice fed n-3 polyunsaturated fatty acids. *The Biochemical journal*, 441(1), pp.359–66. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21880016> [Accessed October 18, 2013].
- Bae, S. & Zhang, L., 2005. Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. *The Journal of pharmacology and experimental therapeutics*, 315(3), pp.1125–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16099927> [Accessed September 11, 2013].
- Baines, C.P., Goto, M. & Downey, J.M., 1997. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J.Mol.Cell Cardiol.*, 29(0022-2828 (Print)), pp.207–216.
- Barp, J. et al., 2002. Myocardial antioxidant and oxidative stress changes due to sex hormones. *Braz.J.Med.Biol.Res.*, 35(0100-879X (Print)), pp.1075–1081.
- Bassani, J.W., Bassani, R.A. & Bers, D.M., 1994. Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *The Journal of physiology*, 476(2), pp.279–93. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1160440&tool=pmcentrez&rendertype=abstract> [Accessed September 12, 2015].
- Becker, L.B. et al., 1999. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am.J.Physiol*, 277(0002-9513 (Print)), pp.H2240–H2246.

- Bell, R.M., Mocanu, M.M. & Yellon, D.M., 2011. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *Journal of molecular and cellular cardiology*, 50(6), pp.940–50. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21385587> [Accessed August 14, 2013].
- Bers, D.M., 2002. Cardiac excitation-contraction coupling. *Nature*, 415(6868), pp.198–205. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11805843> [Accessed July 26, 2014].
- Billman, G.E., Kang, J.X. & Leaf, A., 1997. Prevention of ischemia-induced cardiac sudden death by n-3 polyunsaturated fatty acids in dogs. *Lipids*, 32(0024-4201 (Print)), pp.1161–1168.
- Bognar, Z. et al., 2006. A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death. *Free Radic.Biol.Med.*, 41(0891-5849 (Print)), pp.835–848.
- Bolli, R., 2000. The late phase of preconditioning. *Circ.Res.*, 87(1524-4571 (Electronic)), pp.972–983.
- Bolli, R. & Marbán, E., 1999. Molecular and cellular mechanisms of myocardial stunning. *Physiological reviews*, 79(2), pp.609–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10221990> [Accessed July 23, 2014].
- Brossard, N. et al., 1996. Retroconversion and metabolism of [13C]22:6n-3 in humans and rats after intake of a single dose of [13C]22:6n-3-triacylglycerols. *The American journal of clinical nutrition*, 64(4), pp.577–86. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8839503> [Accessed May 13, 2015].
- Brown, D.A. et al., 2005. Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. *J.Physiol*, 564(0022-3751 (Print)), pp.619–630.
- Burdge, G.C., Jones, A.E. & Wootton, S.A., 2002. Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. *The British journal of nutrition*, 88(4), pp.355–63. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12323085> [Accessed April 14, 2015].
- Burdge, G.C. & Wootton, S.A., 2002. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *The British journal of nutrition*, 88(4), pp.411–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12323090> [Accessed April 19, 2015].
- Burr, M.L. et al., 1989. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet*, 2(0140-6736 (Print)), pp.757–761.

- Castillo, R.L., Arias, C. & Farías, J.G., 2013. Omega 3 chronic supplementation attenuates myocardial ischaemia-reperfusion injury through reinforcement of antioxidant defense system in rats. *Cell biochemistry and function*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24166314> [Accessed December 13, 2013].
- Cawood, A.L. et al., 2010. Eicosapentaenoic acid (EPA) from highly concentrated n-3 fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stability. *Atherosclerosis*, 212(1), pp.252–259. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20542512> [Accessed April 13, 2015].
- Chicco, A.J. et al., 2007. Sex-specific and exercise-acquired cardioprotection is abolished by sarcolemmal KATP channel blockade in the rat heart. *Am.J.Physiol Heart Circ.Physiol*, 292(0363-6135 (Print)), pp.H2432–H2437.
- Childs, C.E. et al., 2012. Changes in rat n-3 and n-6 fatty acid composition during pregnancy are associated with progesterone concentrations and hepatic FADS2 expression. *Prostaglandins, leukotrienes, and essential fatty acids*, 86(4-5), pp.141–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22495065> [Accessed May 11, 2015].
- Clayton, J.A. & Collins, F.S., 2014. Policy: NIH to balance sex in cell and animal studies. *Nature*, 509(7500), pp.282–283. Available at: <http://www.nature.com/news/policy-nih-to-balance-sex-in-cell-and-animal-studies-1.15195> [Accessed January 5, 2015].
- Cottin, S.C., Sanders, T.A. & Hall, W.L., 2011. The differential effects of EPA and DHA on cardiovascular risk factors. *The Proceedings of the Nutrition Society*, 70(2), pp.215–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21349231> [Accessed May 14, 2015].
- Dart, C., 2010. Lipid microdomains and the regulation of ion channel function. *J.Physiol*, 588(1469-7793 (Electronic)), pp.3169–3178.
- Das, D.K. et al., 1992. Preconditioning of heart by repeated stunning. Adaptive modification of antioxidative defense system. *Cell Mol.Biol.(Noisy.-le-grand)*, 38(0145-5680 (Print)), pp.739–749.
- Diep, Q.N., Intengan, H.D. & Schiffrin, E.L., 2000. Endothelin-1 attenuates omega3 fatty acid-induced apoptosis by inhibition of caspase 3. *Hypertension*, 35(0194-911X (Print)), pp.287–291.
- Edwards, A.G. et al., 2009. PKC-permitted elevation of sarcolemmal KATP concentration may explain female-specific resistance to myocardial infarction. *J.Physiol*, 587(1469-7793 (Electronic)), pp.5723–5737.
- Endres, S. et al., 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *The New*

- England journal of medicine*, 320(5), pp.265–71. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2783477> [Accessed July 19, 2014].
- Van Eyk, J.E. et al., 1998. Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. *Circulation research*, 82(2), pp.261–71. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9468197> [Accessed September 12, 2015].
- Fan, Y.-Y. et al., 2004. Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production. *Journal of immunology (Baltimore, Md. : 1950)*, 173(10), pp.6151–60. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15528352> [Accessed July 20, 2014].
- Farina, M. et al., 2003. In vitro effects of selenite and mercuric chloride on liver thiobarbituric acid-reactive substances and non-protein thiols from rats: influences of dietary cholesterol and polyunsaturated and saturated fatty acids. *Nutrition*, 19(0899-9007 (Print)), pp.531–535.
- Feng, G.M. et al., 2012. Effect of docosahexaenoic acid on hypoxia/reoxygenation injury in human coronary arterial smooth muscle cells. *Eur.J.Nutr.*, 51(1436-6215 (Electronic)), pp.987–995.
- Folch, J., Lees, M. & Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J.Biol.Chem.*, 226(0021-9258 (Print)), pp.497–509.
- Forbes, R.A., Steenbergen, C. & Murphy, E., 2001. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ.Res.*, 88(1524-4571 (Electronic)), pp.802–809.
- Force, T. et al., 1989. n-3 fatty acids increase postischemic blood flow but do not reduce myocardial necrosis. *Am.J.Physiol*, 257(0002-9513 (Print)), pp.H1204–H1210.
- Ford, D.A. et al., 1991. The rapid and reversible activation of a calcium-independent plasmalogen-selective phospholipase A2 during myocardial ischemia. *The Journal of clinical investigation*, 88(1), pp.331–5. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=296037&tool=pmcentrez&rendertype=abstract> [Accessed July 21, 2014].
- Freeman, B.A. & Crapo, J.D., 1982. Biology of disease: free radicals and tissue injury. *Lab Invest*, 47(0023-6837 (Print)), pp.412–426.
- Frøyland, L. et al., 1996. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs. *Lipids*, 31(2), pp.169–78. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8835405> [Accessed May 14, 2015].

- Gallagher, K.P. et al., 1986. Failure of superoxide dismutase and catalase to alter size of infarction in conscious dogs after 3 hours of occlusion followed by reperfusion. *Circulation*, 73(0009-7322 (Print)), pp.1065–1076.
- Gao, L. et al., 2014. Degradation of cardiac myosin light chain kinase by matrix metalloproteinase-2 contributes to myocardial contractile dysfunction during ischemia/reperfusion. *Journal of molecular and cellular cardiology*, 77, pp.102–12. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25451385> [Accessed September 12, 2015].
- Garcia-Alonso, F.J. et al., 2012. Effect of consumption of tomato juice enriched with n-3 polyunsaturated fatty acids on the lipid profile, antioxidant biomarker status, and cardiovascular disease risk in healthy women. *Eur.J.Nutr.*, 51(1436-6215 (Electronic)), pp.415–424.
- Gawrisch, K., Eldho, N. V & Holte, L.L., 2003. The structure of DHA in phospholipid membranes. *Lipids*, 38(0024-4201 (Print)), pp.445–452.
- Gordon, A.M., Homsher, E. & Regnier, M., 2000. Regulation of contraction in striated muscle. *Physiological reviews*, 80(2), pp.853–924. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10747208> [Accessed September 28, 2015].
- Gottlieb, R.A. et al., 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *The Journal of clinical investigation*, 94(4), pp.1621–8. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=295322&tool=pmcentrez&rendertype=abstract> [Accessed November 9, 2013].
- Halestrap, A.P., Clarke, S.J. & Javadov, S.A., 2004. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovasc.Res.*, 61(0008-6363 (Print)), pp.372–385.
- Halliwell, B. & Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. *Am.J.Clin.Nutr.*, 57(0002-9165 (Print)), p.715S–724S.
- Hamacher-Brady, A., Brady, N.R. & Gottlieb, R.A., 2006. The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy*, 20(6), pp.445–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17149555> [Accessed September 12, 2015].
- Hanwell, H.E.C. et al., 2009. Acute fish oil and soy isoflavone supplementation increase postprandial serum (n-3) polyunsaturated fatty acids and isoflavones but do not affect triacylglycerols or biomarkers of oxidative stress in overweight and obese hypertriglyceridemic men. *The Journal of nutrition*, 139(6), pp.1128–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19339704> [Accessed June 26, 2014].

- Harris, W.S. et al., 2013. Comparative effects of an acute dose of fish oil on omega-3 fatty acid levels in red blood cells versus plasma: implications for clinical utility. *Journal of clinical lipidology*, 7(5), pp.433–40. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24079284> [Accessed November 12, 2013].
- Health Canada, 2009. No Title. *Health Portfolio Sex and Gender-Based Analysis Policy*. Available at: <http://www.hc-sc.gc.ca/hl-vs/pubs/women-femmes/sgba-policy-politique-ags-eng.php> [Accessed April 19, 2015].
- Hodge, J., Sanders, K. & Sinclair, A.J., 1993. Differential utilization of eicosapentaenoic acid and docosahexaenoic acid in human plasma. *Lipids*, 28(6), pp.525–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8355578> [Accessed May 14, 2015].
- Vanden Hoek, T.L. et al., 1998. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J.Biol.Chem.*, 273(0021-9258 (Print)), pp.18092–18098.
- Hoffman, J.W. et al., 2004. Myocardial reperfusion injury: etiology, mechanisms, and therapies. *The Journal of extra-corporeal technology*, 36(4), pp.391–411. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15679285> [Accessed July 23, 2014].
- Hoshida, S. et al., 1993. Sublethal ischemia alters myocardial antioxidant activity in canine heart. *Am.J.Physiol*, 264(0002-9513 (Print)), pp.H33–H39.
- Ibrahim, W. et al., 1999. Oxidative stress and antioxidant status in mouse kidney: effects of dietary lipid and vitamin E plus iron. *J.Nutr.Biochem.*, 10(0955-2863 (Print)), pp.674–678.
- Ikonomidis, J.S. et al., 1997. Preconditioning cultured human pediatric myocytes requires adenosine and protein kinase C. *Am.J.Physiol*, 272(0002-9513 (Print)), pp.H1220–H1230.
- Imahashi, K. et al., 2005. Cardiac-specific ablation of the Na⁺-Ca²⁺ exchanger confers protection against ischemia/reperfusion injury. *Circulation research*, 97(9), pp.916–21. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16179590> [Accessed September 12, 2015].
- Ishikado, A. et al., 2013. 4-Hydroxy hexenal derived from docosahexaenoic acid protects endothelial cells via Nrf2 activation. *PloS one*, 8(7), p.e69415. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3720569&tool=pmcentrez&rendertype=abstract> [Accessed May 11, 2015].
- Jahangiri, A. et al., 2006. Dietary fish oil alters cardiomyocyte Ca²⁺ dynamics and antioxidant status. *Free Radic.Biol.Med.*, 40(0891-5849 (Print)), pp.1592–1602.
- Javouhey, A., Rochette, L. & Rocquelin, G., 1989. In vitro study of functional parameters and cardiac arrhythmia in localized ischemia followed by reperfusion in two rat strains (Sprague Dawley and Wistar). *Comptes rendus des séances de la Société de biologie et de ses filiales*,

- 183(6), pp.530–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2534957> [Accessed November 5, 2013].
- Johnson, M.S., Moore, R.L. & Brown, D.A., 2006. Sex differences in myocardial infarct size are abolished by sarcolemmal KATP channel blockade in rat. *Am.J.Physiol Heart Circ.Physiol*, 290(0363-6135 (Print)), pp.H2644–H2647.
- Jones, S.P. et al., 2003. Role of intracellular antioxidant enzymes after in vivo myocardial ischemia and reperfusion. *Am.J.Physiol Heart Circ.Physiol*, 284(0363-6135 (Print)), pp.H277–H282.
- Kain, V. et al., 2015. Resolvin D1 activates the inflammation resolving response at splenic and ventricular site following myocardial infarction leading to improved ventricular function. *Journal of molecular and cellular cardiology*, 84, pp.24–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25870158> [Accessed May 12, 2015].
- Kain, V., Prabhu, S.D. & Halade, G. V, 2014. Inflammation revisited: inflammation versus resolution of inflammation following myocardial infarction. *Basic research in cardiology*, 109(6), p.444. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25248433> [Accessed May 12, 2015].
- Kalogeris, T. et al., 2012. Cell biology of ischemia/reperfusion injury. *International review of cell and molecular biology*, 298, pp.229–317. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3904795&tool=pmcentrez&rendertype=abstract> [Accessed September 13, 2015].
- Kang, J.X. & Leaf, A., 1994. Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. *Proc.Natl.Acad.Sci.U.S.A*, 91(0027-8424 (Print)), pp.9886–9890.
- Kang, J.X., Xiao, Y.F. & Leaf, A., 1995. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 92(9), pp.3997–4001. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=42089&tool=pmcentrez&render type=abstract> [Accessed July 21, 2014].
- Kelley, D.S. et al., 1999. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. *Lipids*, 34(4), pp.317–24. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10443964> [Accessed July 15, 2014].
- Kennedy, E.P. & Weiss, S.B., 1956. The function of cytidine coenzymes in the biosynthesis of phospholipides. *The Journal of biological chemistry*, 222(1), pp.193–214. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/13366993> [Accessed July 12, 2014].

- Kevin, L.G. et al., 2003. Ischemic preconditioning alters real-time measure of O₂ radicals in intact hearts with ischemia and reperfusion. *Am.J.Physiol Heart Circ.Physiol*, 284(0363-6135 (Print)), pp.H566–H574.
- Khanamiri, S. et al., 2013. Contribution of Kv7 channels to basal coronary flow and active response to ischemia. *Hypertension*, 62(6), pp.1090–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24082059> [Accessed September 12, 2015].
- Kim, J.S., Jin, Y. & Lemasters, J.J., 2006. Reactive oxygen species, but not Ca²⁺ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *Am.J.Physiol Heart Circ.Physiol*, 290(0363-6135 (Print)), pp.H2024–H2034.
- Kitson, A.P. et al., 2012. Tissue-specific sex differences in docosahexaenoic acid and Delta6-desaturase in rats fed a standard chow diet. *Appl.Physiol Nutr.Metab*, 37(1715-5312 (Print)), pp.1200–1211.
- Kubo, K. et al., 2000. Preferential incorporation of docosahexaenoic acid into nonphosphorus lipids and phosphatidylethanolamine protects rats from dietary DHA-stimulated lipid peroxidation. *The Journal of nutrition*, 130(7), pp.1749–59. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10867046> [Accessed May 14, 2015].
- Kwak, S.M. et al., 2012. Efficacy of omega-3 fatty acid supplements (eicosapentaenoic acid and docosahexaenoic acid) in the secondary prevention of cardiovascular disease: a meta-analysis of randomized, double-blind, placebo-controlled trials. *Arch.Intern.Med.*, 172(1538-3679 (Electronic)), pp.686–694.
- Lagranha, C.J. et al., 2010. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ.Res.*, 106(1524-4571 (Electronic)), pp.1681–1691.
- Lands, W.E., 1958. Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis. *The Journal of biological chemistry*, 231(2), pp.883–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/13539023> [Accessed July 12, 2014].
- Lee, J.Y. et al., 2003. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *Journal of lipid research*, 44(3), pp.479–86. Available at: <http://www.jlr.org.proxy.lib.uwaterloo.ca/content/44/3/479.long> [Accessed July 20, 2014].
- Maddika, S. et al., 2009. Role of oxidative stress in ischemia-reperfusion-induced alterations in myofibrillar ATPase activities and gene expression in the heart. *Canadian journal of physiology and pharmacology*, 87(2), pp.120–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19234575> [Accessed July 27, 2014].

- Malloy, C.R. et al., 1990. Influence of global ischemia on intracellular sodium in the perfused rat heart. *Magnetic Resonance in Medicine : Official Journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*, 15(1), pp.33–44. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2374498>.
- Maulik, N. et al., 1995. Myocardial adaptation to ischemia by oxidative stress induced by endotoxin. *Am.J.Physiol*, 269(0002-9513 (Print)), pp.C907–C916.
- McGuinness, J. et al., 2006. Myocardial protection using an omega-3 fatty acid infusion: quantification and mechanism of action. *J.Thorac.Cardiovasc.Surg.*, 132(1097-685X (Electronic)), pp.72–79.
- McLennan, P.L. et al., 1993. Comparative efficacy of n-3 and n-6 polyunsaturated fatty acids in modulating ventricular fibrillation threshold in marmoset monkeys. *Am.J.Clin.Nutr.*, 58(0002-9165 (Print)), pp.666–669.
- McLennan, P.L., Abeywardena, M.Y. & Charnock, J.S., 1985. Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Can.J.Physiol Pharmacol.*, 63(0008-4212 (Print)), pp.1411–1417.
- Meissner, G. & Lu, X., 1995. Dihydropyridine receptor-ryanodine receptor interactions in skeletal muscle excitation-contraction coupling. *Bioscience reports*, 15(5), pp.399–408. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8825041> [Accessed September 28, 2015].
- Metherel, A.H. et al., 2009. Assessment of blood measures of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women. *Prostaglandins, leukotrienes, and essential fatty acids*, 81(1), pp.23–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19515545> [Accessed May 12, 2015].
- Metherel, A.H. et al., 2012. Omega-3 polyunsaturated fatty acid profiling using fingertip-prick whole blood does not require overnight fasting before blood collection. *Nutrition research (New York, N.Y.)*, 32(8), pp.547–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22935337> [Accessed May 13, 2015].
- Mori, T.A. et al., 1999. Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. *Hypertension*, 34(2), pp.253–60. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10454450> [Accessed May 14, 2015].
- Morrison, W.R. & Smith, L.M., 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron-fluoride-methanol. *J.Lipid Res.*, 5(0022-2275 (Print)), pp.600–608.
- Mozaffarian, D. & Wu, J.H., 2011. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *J.Am.Coll.Cardiol.*, 58(1558-3597 (Electronic)), pp.2047–2067.

- Murphy, E. et al., 1991. Amiloride delays the ischemia-induced rise in cytosolic free calcium. *Circulation research*, 68(5), pp.1250–1258.
- Murphy, E. & Steenbergen, C., 2008. Ion transport and energetics during cell death and protection. *Physiology (Bethesda, Md.)*, 23, pp.115–23. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2872775&tool=pmcentrez&rendertype=abstract> [Accessed September 19, 2015].
- Murphy, E. & Steenbergen, C., 2008. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev.*, 88(0031-9333 (Print)), pp.581–609.
- Murry, C.E., Jennings, R.B. & Reimer, K.A., 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*, 74(0009-7322 (Print)), pp.1124–1136.
- Nakagawa, F. et al., 2014. 4-Hydroxy hexenal derived from dietary n-3 polyunsaturated fatty acids induces anti-oxidative enzyme heme oxygenase-1 in multiple organs. *Biochemical and biophysical research communications*, 443(3), pp.991–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24361890> [Accessed May 11, 2015].
- Nakano, A., Cohen, M. V & Downey, J.M., 2000. Ischemic preconditioning: from basic mechanisms to clinical applications. *Pharmacol.Ther.*, 86(0163-7258 (Print)), pp.263–275.
- Ohnishi, H. & Saito, Y., 2013. Eicosapentaenoic acid (EPA) reduces cardiovascular events: relationship with the EPA/arachidonic acid ratio. *Journal of atherosclerosis and thrombosis*, 20(12), pp.861–77. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24047614> [Accessed May 13, 2015].
- Olivecrona, G.K. et al., 2007. Mild hypothermia reduces cardiac post-ischemic reactive hyperemia. *BMC cardiovascular disorders*, 7, p.5. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1808476&tool=pmcentrez&rendertype=abstract> [Accessed September 12, 2015].
- Ooiwa, H. et al., 1991. Superoxide dismutase conjugated to polyethylene glycol fails to limit myocardial infarct size after 30 min ischemia followed by 72 h of reperfusion in the rabbit. *J.Mol.Cell Cardiol.*, 23(0022-2828 (Print)), pp.119–125.
- Opie, L.H., 1989. Reperfusion injury and its pharmacologic modification. *Circulation*, 80(0009-7322 (Print)), pp.1049–1062.
- Pagliaro, P. et al., 2001. Ischemic preconditioning: from the first to the second window of protection. *Life Sci.*, 69(0024-3205 (Print)), pp.1–15.
- Patterson, A.C. et al., 2014. The percentage of DHA in erythrocytes can detect non-adherence to advice to increase EPA and DHA intakes. *The British journal of nutrition*, 111(2), pp.270–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23920312> [Accessed July 2, 2014].

- Pawlosky, R. et al., 2003. n-3 fatty acid metabolism in women. *The British journal of nutrition*, 90(5), pp.993–4; discussion 994–5. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14667193> [Accessed April 19, 2015].
- Penna, C. et al., 2009. Cardioprotection: a radical view Free radicals in pre and postconditioning. *Biochim.Biophys.Acta*, 1787(0006-3002 (Print)), pp.781–793.
- Pike, M.M., Kitakaze, M. & Marban, E., 1990. ²³Na-NMR measurements of intracellular sodium in intact perfused ferret hearts during ischemia and reperfusion. *The American journal of physiology*, 259(6 Pt 2), pp.H1767–H1773.
- Piper, H.M., Meuter, K. & Schäfer, C., 2003. Cellular mechanisms of ischemia-reperfusion injury. *The Annals of thoracic surgery*, 75(2), pp.S644–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12607706> [Accessed August 15, 2013].
- Raatz, S.K. et al., 2009. Enhanced absorption of n-3 fatty acids from emulsified compared with encapsulated fish oil. *Journal of the American Dietetic Association*, 109(6), pp.1076–81. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2701654&tool=pmcentrez&rendertype=abstract> [Accessed June 27, 2014].
- Reeve, J.L. et al., 2007. Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by Bcl-xL. *J.Cell Mol.Med.*, 11(1582-1838 (Print)), pp.509–520.
- Researchers, G.P., 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet*, 354(0140-6736 (Print)), pp.447–455.
- Richard, D. et al., 2014. Infusion of docosahexaenoic acid protects against myocardial infarction. *Prostaglandins, leukotrienes, and essential fatty acids*, 90(4), pp.139–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24480264> [Accessed May 9, 2015].
- Rizos, E.C. et al., 2012. Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. *JAMA*, 308(1538-3598 (Electronic)), pp.1024–1033.
- Rodrigo, R., Prieto, J.C. & Castillo, R., 2013. Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical applications. *Clin.Sci.(Lond)*, 124(1470-8736 (Electronic)), pp.1–15.
- Rodrigues, P.O. et al., 2014. Influence of feeding graded levels of canned sardines on the inflammatory markers and tissue fatty acid composition of Wistar rats. *The British journal of nutrition*, 112(3), pp.309–19. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24775714> [Accessed May 13, 2015].

- Rudiger, H.A., Graf, R. & Clavien, P.A., 2003. Sub-lethal oxidative stress triggers the protective effects of ischemic preconditioning in the mouse liver. *J.Hepatol.*, 39(0168-8278 (Print)), pp.972–977.
- Saito, M. & Kubo, K., 2003. Relationship between tissue lipid peroxidation and peroxidizability index after alpha-linolenic, eicosapentaenoic, or docosahexaenoic acid intake in rats. *Br.J.Nutr.*, 89(0007-1145 (Print)), pp.19–28.
- Scherer, N.M. & Deamer, D.W., 1986. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca²⁺-ATPase. *Archives of biochemistry and biophysics*, 246(2), pp.589–601. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2939799> [Accessed July 27, 2014].
- Schlüter, K.D. et al., 1996. Protection of reoxygenated cardiomyocytes against osmotic fragility by nitric oxide donors. *The American journal of physiology*, 271(2 Pt 2), pp.H428–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8770080> [Accessed July 27, 2014].
- Schwarz, E.R. et al., 2000. What is the required reperfusion period for assessment of myocardial infarct size using triphenyltetrazolium chloride staining in the rat? *Journal of thrombosis and thrombolysis*, 10(2), pp.181–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11005940> [Accessed June 25, 2014].
- Serhan, C.N. et al., 2000. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *The Journal of experimental medicine*, 192(8), pp.1197–204. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2195872&tool=pmcentrez&rendertype=abstract> [Accessed July 19, 2014].
- Serhan, C.N., 2007. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annual review of immunology*, 25, pp.101–37. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17090225> [Accessed July 9, 2014].
- Sheu, S.S., Nauduri, D. & Anders, M.W., 2006. Targeting antioxidants to mitochondria: a new therapeutic direction. *Biochim.Biophys.Acta*, 1762(0006-3002 (Print)), pp.256–265.
- Sibbons, C.M. et al., Effect of sex hormones on n-3 polyunsaturated fatty acid biosynthesis in HepG2 cells and in human primary hepatocytes. *Prostaglandins, leukotrienes, and essential fatty acids*, 90(2-3), pp.47–54. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4046896&tool=pmcentrez&rendertype=abstract> [Accessed May 11, 2015].
- Sindram, D. et al., 2002. Ischemic preconditioning protects against cold ischemic injury through an oxidative stress dependent mechanism. *J.Hepatol.*, 36(0168-8278 (Print)), pp.78–84.

- Skrzypiec-Spring, M. et al., 2006. Isolated heart perfusion according to Langendorff---still viable in the new millennium. *Journal of pharmacological and toxicological methods*, 55(2), pp.113–26. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16844390> [Accessed August 15, 2013].
- Smith, G.L. et al., 1993. Relationship between intracellular pH and metabolite concentrations during metabolic inhibition in isolated ferret heart. *The Journal of physiology*, 472, pp.11–22.
- Smith, T.L., Rush, J.W.E. & Stark, K.D., 2012. The infusion of docosahexaenoic acid during reperfusion after ischemia has dose dependent effects in isolated perfused rat hearts. *International Society for the Study of Fatty Acids and Lipids*.
- Song, J.H., Fujimoto, K. & Miyazawa, T., 2000. Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. *J.Nutr.*, 130(0022-3166 (Print)), pp.3028–3033.
- Song, X. et al., 2003. Effects of sex, gonadectomy, and oestrogen substitution on ischaemic preconditioning and ischaemia-reperfusion injury in mice. *Acta Physiol Scand.*, 177(0001-6772 (Print)), pp.459–466.
- De Souza, M. das G.C. et al., 2015. n-3 PUFA induce microvascular protective changes during ischemia/reperfusion. *Lipids*, 50(1), pp.23–37. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4282880&tool=pmcentrez&rendertype=abstract> [Accessed May 9, 2015].
- Stark, K.D. & Holub, B.J., 2004. Differential eicosapentaenoic acid elevations and altered cardiovascular disease risk factor responses after supplementation with docosahexaenoic acid in postmenopausal women receiving and not receiving hormone replacement therapy. *The American journal of clinical nutrition*, 79(5), pp.765–73. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15113713> [Accessed May 13, 2015].
- Steenbergen, C. et al., 1987. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circulation research*, 60(5), pp.700–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3109761> [Accessed September 12, 2015].
- Stillwell, W. & Wassall, S.R., 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chemistry and physics of lipids*, 126(1), pp.1–27. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14580707> [Accessed July 15, 2014].
- Sun, J.Z. et al., 1996. Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J.Clin.Invest*, 97(0021-9738 (Print)), pp.562–576.

- Suzuki, S. et al., 1991. Alterations in cardiac contractile proteins due to oxygen free radicals. *Biochimica et biophysica acta*, 1074(1), pp.95–100. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1646033> [Accessed July 27, 2014].
- Tang, X.L. et al., 2000. Ischemic preconditioning does not upregulate antioxidant enzymes in conscious rabbits. *J.Mol.Cell Cardiol.*, 32, pp.A54–A54.
- Tang, X.L. et al., 1997. Late preconditioning against stunning is not mediated by increased antioxidant defenses in conscious pigs. *Am.J.Physiol*, 273(0002-9513 (Print)), pp.H1651–H1657.
- Temsah, R.M. et al., 1999. Alterations in sarcoplasmic reticulum function and gene expression in ischemic-reperfused rat heart. *The American journal of physiology*, 277(2 Pt 2), pp.H584–94. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10444483> [Accessed July 27, 2014].
- Tritto, I. et al., 1997. Oxygen radicals can induce preconditioning in rabbit hearts. *Circ.Res.*, 80(0009-7330 (Print)), pp.743–748.
- Turcato, S. et al., 2006. Ischemic preconditioning depends on age and gender. *Basic Res.Cardiol.*, 101(0300-8428 (Print)), pp.235–243.
- Vanhaecke, J. et al., 1991. Effect of superoxide dismutase on infarct size and postischemic recovery of myocardial contractility and metabolism in dogs. *J.Am.Coll.Cardiol.*, 18(0735-1097 (Print)), pp.224–230.
- Vossen, R.C. et al., 1995. Differential effects of endothelial cell fatty acid modification on the sensitivity of their membrane phospholipids to peroxidation. *Prostaglandins Leukot.Essent.Fatty Acids*, 52(0952-3278 (Print)), pp.341–347.
- Wang, Q.D., Swärdh, A. & Sjöquist, P.O., 2001. Relationship between ischaemic time and ischaemia/reperfusion injury in isolated Langendorff-perfused mouse hearts. *Acta physiologica Scandinavica*, 171(2), pp.123–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11350272> [Accessed September 10, 2013].
- Wong, S.W. et al., 2009. Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *The Journal of biological chemistry*, 284(40), pp.27384–92. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2785667&tool=pmcentrez&rendertype=abstract> [Accessed July 20, 2014].
- Xiao, Y.F. et al., 2001. Single point mutations affect fatty acid block of human myocardial sodium channel alpha subunit Na⁺ channels. *Proc.Natl.Acad.Sci.U.S.A*, 98(0027-8424 (Print)), pp.3606–3611.

- Xiao, Y.F. et al., 1997. Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. *Proc.Natl.Acad.Sci.U.S.A*, 94(0027-8424 (Print)), pp.4182–4187.
- Yamashita, N. et al., 1999. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *J.Exp.Med.*, 189(0022-1007 (Print)), pp.1699–1706.
- Yamashita, N. et al., 1994. Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. *J.Clin.Invest*, 94(0021-9738 (Print)), pp.2193–2199.
- Yamashita, N. et al., 1998. Whole-body hyperthermia provides biphasic cardioprotection against ischemia/reperfusion injury in the rat. *Circulation*, 98(0009-7322 (Print)), pp.1414–1421.
- Yang, Z.-H. et al., 2012. Ingestion of a single serving of saury alters postprandial levels of plasma n-3 polyunsaturated fatty acids and long-chain monounsaturated fatty acids in healthy human adults. *Lipids in health and disease*, 11, p.95. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3485116&tool=pmcentrez&rendertype=abstract> [Accessed May 23, 2014].
- Zeghichi-Hamri, S. et al., 2010. Protective effect of dietary n-3 polyunsaturated fatty acids on myocardial resistance to ischemia-reperfusion injury in rats. *Nutr.Res.*, 30(1879-0739 (Electronic)), pp.849–857.
- Zhang, Y. et al., 2010. 4-hydroxy-2-nonenal protects against cardiac ischemia-reperfusion injury via the Nrf2-dependent pathway. *Journal of molecular and cellular cardiology*, 49(4), pp.576–86. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20685357> [Accessed August 16, 2013].
- Zweier, J. & Talukder, M., 2006. The role of oxidants and free radicals in reperfusion injury. *Cardiovascular Research*, 70(2), pp.181–190. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16580655> [Accessed September 18, 2015].

Appendix



Figure 26. Representative Ponceau S stain demonstrating equal protein loading of polyvinylidene difluoride membrane used for chemiluminescent analysis of CuZnSOD protein levels in hearts from male Wistar rats.

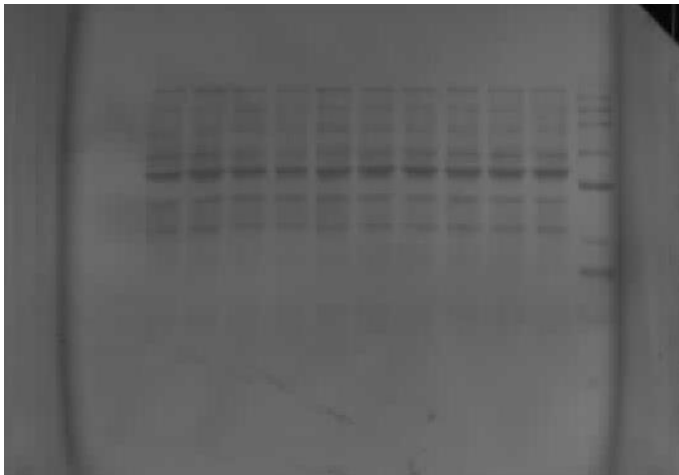


Figure 27. Representative Ponceau S stain demonstrating equal protein loading of polyvinylidene difluoride membrane used for chemiluminescent analysis of MnSOD protein levels in hearts from male Wistar rats.



Figure 28. Representative Ponceau S stain demonstrating equal protein loading of polyvinylidene difluoride membrane used for chemiluminescent analysis of GPx protein levels in hearts from male Wistar rats.